Saskatchewan
Agriculture, Food
and Rural
Revitalization

ADF

AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

19990364

DEVELOPMENT OF PATHOGENIC FUNGI AS BIOHERBICIDES FOR CONTROL OF CLEAVERS AND CHICKWEED

Funded by:
The Agriculture Development Fund

March 2003
Prepared by: Alberta Research Council

ALBERTA RESEARCH COUNCIL

FINAL REPORT

AGRICULTURE DEVELOPMENT FUND
RECEIVED
MAR 12 2003
REGINA, SASK.
S4S 0E1
PROVINCE of SASKATCHEWAN

Saskatchewan ADF Research Project # 19990364
Alberta Agricultural Research Institute Research project #1999M528

(February 2003)

Development of Bioherbicides For Biological Control of Cleavers

Wenming Zhang[1], Karen Bailey[2], and Dan Cole[3]

[1]Alberta Research Council, PO Bag 4000, Vegreville, AB T9C 1T4

[2]Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, SK S7N 0X2

[3] Alberta Agriculture, Food, and Rural Development, Edmonton, AB T6H 4P2

Executive Summary

Objectives of this project have successfully been achieved. A native fungal pathogen *Plectosporium tabacinum* has been identified as a potential bioherbicide product for the control of cleavers. Both US and Canadian patent have been applied for (No. 20020177528 and No. 2,325,215). A naturally occurring bacterium *Burkholderia andropogonis* has also been identified as a potential bioherbicide for control of chickweed. A provisional patent has been applied for. Four refereed journal articles, one proceeding paper, and three posters have been generated from this study. Further development of both bioherbicides as weed control products requires the study on fermentation, formulation, and integration into current weed control system.

Acknowledgements

Funding from the Alberta Agricultural Research Institute, Saskatchewan Agriculture Development fund, and the Canadian Seed Growers' Association are greatly appreciated. Technical assistances from Sandi Robertson, Michelle Sulz, Tanya Mykietiak, and Kevin McKee are immensely appreciated. Authors also thank Dr. L. M. Hall and K. Topinka at the Alberta Agriculture, Food and Rural Development, Edmonton, AB and Dr. A. S. McClay at the Alberta Research Council, Vegreville, AB for providing various types of weed and crop seeds. Special thanks to Jay Yanke at the Agriculture and Agri-Food Canada, Lethbridge Research Centre, Lethbridge, AB for characterizing and identifying the bacterium. Another thanks to Dr. A. G. Thomas, Agriculture and Agri-Food Canada, Saskatoon, for his suggestions on target weed selection in our biocontrol program and for providing weed distribution maps of western Canada. Editorial assistance from Lorraine Yakemchuk is also greatly appreciated.

Table of Contents

PAGE

EXECUTIVE SUMMARY i

ACKNOWLEDGEMENTS ii

LIST OF TABLES x

LIST OF FIGURES xii

PART I - CLEAVERS BIOHERBICIDE 1

Summary 2

CHAPTER 1. BACKGROUND INFORMATION 4

Literature Cited 5

CHAPTER 2 GROWTH AND SPORE PRODUCTION OF *PLECTOSPORIUM TABACINUM* 6

2.1. Abstract 6

2.2. Introduction 6

2.3. Materials And Methods 8

2.3.1. General Experimental Procedure. 8

2.3.2. Mycelial Growth, Sporulation, and Spore Germination On Standard Agar Media 10

2.3.2.1 Effect Of Culture Media and Light Regime. 10

2.3.2.2 Effect Of PDA Strength and Light Regime 11

2.3.2.3 Effect Of pH. 11

2.3.2.4 Effect Of Temperature. 12

2.3.3. Spore Production In Submerged Liquid Cultures 12

2.3.3.1. Effect Of Liquid Culture Media. 12

2.3.3.2. Effect Of Osmotic Potential. 12

2.3.3.3. Effect Of Initial Spore Concentration. 12

2.3.3.4. Spore Production Over Incubation Time. 13

2.3.3.5. Effect Of Nitrogen Source 13

2.3.3.6. Effect Of Carbon Source. 13

2.3.3.7. Effect Of Carbon Concentration. 14

2.3.3.8. Effect Of Carbon-To-Nitrogen (Cn) Ratio. 14

2.4. Results 14

2.4.1. Mycelial Growth, Sporulation, Spore Germination On Standard Agar Media 14

2.4.1.1. Effect Of Culture Media and Light Regime. 14

2.4.1.2. Effect Of Pda Strength and Light Regime 14

2.4.1.3. Effect Of pH. 15

2.4.1.4. Effect Of Temperature. 15

2.4.2. Spore Production In Submerged Liquid Cultures 16
2.4.2.1. Effect Of Liquid Culture Media. 16
2.4.2.2. Effect Of Osmotic Potential. 16
2.4.2.3. Effect Of Initial Spore Concentration. 16
2.4.2.4. Spore Production Over Incubation Time. 16
2.4.2.5. Effect Of Nitrogen Source 17
2.4.2.6. Effect Of Carbon Source. 17
2.4.2.7. Effect Of Carbon Concentration. 18
2.4.2.8. Effect Of Carbon-To-Nitrogen (C:N) Ratio. 18
2.5. Discussion 19
2.6. Literature Cited 21
CHAPTER 3 HOST RANGE OF *PLECTOSPORIUM TABACINUM* 38
3.1. Abstract 38
3.2. Introduction 38
3.3. Materials And Methods 39
3.3.1. The Pathogen 39
3.3.2. Inoculum Production. 40
3.3.3. Pathogenicity Of *P. Tabacinum* To False Cleavers and Canola. 40
3.3.4. Host Range Of *P. Tabacinum*. 41
3.4. Results 42
3.4.1. Pathogenicity Of *P. Tabacinum* To False Cleavers and Canola. 42
3.4.2. Host Range Of *P. Tabacinum*. 42
3.5. Discussions 43
3.6. Literature Cited 44
CHAPTER 4 DETERMINATION OF OPTIMUM CONDITIONS AND LIMITING FACTORS FOR MAXIMUM EFFECTIVENESS OF *PLECTOSPORIUM TABACINUM* FOR CONTROL OF CLEAVERS (*GALIUM SPURIUM*) 52
4.1. Abstract 52
4.2. Introduction 52
4.3. Materials And Methods 53
4.3.1. Inoculum Production. 53
4.3.2. Plant Production. 54
4.3.3. Inoculation Procedure. 54
4.3.4. Assessment Of Disease Development. 54
4.3.5. Effect Of Inoculum Concentration and Plant Growth Stage. 54

4.3.6. Effect Of Dew Period Duration....55
4.3.7. Interaction Of Inoculum Concentration and Dew Period Duration. 55
4.3.8. Interaction Of Inoculum Concentration and Dew Period Temperature. 55
4.3.9. Effect Of Repetitive Dew....55
4.3.10. Effect Of Delaying Initial Dew Period....55
4.3.11. Data Analysis. 56
4.4. Results 56
4.4.1. Effect Of Inoculum Concentration and Plant Growth Stage. 56
4.4.2. Effect Of Dew Period Duration....56
4.4.3. Interaction Of Inoculum Concentration and Dew Period Duration. 57
4.4.4. Interaction Of Dew Period Temperature and Inoculum Concentration. 57
4.4.5. Effect Of Repetitive Dew Periods. 57
4.4.6. Effect Of Delaying Initial Dew Period....57
4.5. Discussion 58
4.6. Literature Cited 59
CHAPTER 5 FIELD EFFICACY OF CLEAVERS CONTROL WITH *PLECTOSPORIUM TABACINUM* 66
5.1. Abstract 66
5.2. Introduction 66
5.3. Materials and Methods 66
5.3.1. Plant Production. 66
5.3.2. Spore Production. 67
5.3.3. Culture Filtrates Preparation. 67
5.3.4. Efficacy With Fungal Spores. 67
5.3.5 Efficacy With Culture Filtrates. 68
5.3.6. Data Analyses....68
5.4. Results 68
5.4.1. Efficacy With Fungal Spores. 68
5.4.2. Efficacy With Culture Filtrates. 68
5.5. Discussions....69

PART II - CHICKWEED BIOHERBICIDE....74
Summary 75
CHAPTER 1. BACKGROUND INFORMATION....76
Literature Cited 77

CHAPTER 2 COLLECTION AND SCREENING OF BIOHERBICIDE CANDIDATES FOR CONTROL OF CHICKWEED 78
2.1. Abstract 78
2.2 Introduction 78
2.3. Materials and Methods 79
2.3.1. Collection and Isolation Of Fungi And Bacteria 79
2.3.1.1. Collection Of Diseased Chickweed. 79
2.3.1.2. Isolation Of Causal Organisms 79
2.3.2. Maintenance Of Microorganisms 79
2.3.3. Screening Of Fungi And Bacteria 80
2.3.3.1. Inoculum Preparation 80
2.3.3.2. Plant Production 81
2.3.3.3. Inoculation Procedure 81
2.3.3.4. Pathogenicity And Virulence To Chickweed 81
2.3.4. Disease Symptoms Of CW00B006C In Chickweed 82
2.4. Results And Discussions 82
2.4.1. Collection and Isolation Of Fungi And Bacteria 82
2.4.2. Screening Of Fungi and Bacteria. 82
2.4.3. Disease Symptoms Of CW00B006C In Chickweed 83
2.5. Literature Cited 83
CHAPTER 3 CHARACTERIZATION AND IDENTIFICATION OF BACTERIUM CW00B006C 88
3.1. Abstract 88
3.2. Introduction 88
3.3. Materials And Methods 88
3.3.1. Phenotypic Analyses 88
3.3.1.1. Physiological and Biochemical Characterization 88
3.3.1.2. Fatty Acid Composition (Midi-Fame) 89
3.3.2. Genotypic Analyses (16S rDNA Sequence) 89
3.4. Results And Discussions 89
3.4.1. Phenotypic Analyses 89
3.4.1.1. Physiological and Biochemical Characterization 89
3.4.1.2. Fatty Acid Composition 90
3.4.2. Genotypic Analyses (16S rDNA Sequencing) 90
3.5 Literature Cited 91

CHAPTER 4 CELL PRODUCTION OF BACTERIUM CW00B006C 95
4.1. Abstract 95
4.2. Introduction 95
4.3. Materials and Methods 95
4.3.1. Growth Curve Of The Bacterium In Different Liquid Culture Media 96
4.3.2. Effect Of Medium pH On Bacterial Cell Production 97
4.3.3. Effect Of Growth Medium Modification With Chickweed Extracts. 97
4.4. Results And Discussions 98
4.4.1. Growth Curve Of The Bacterium In Four Different Liquid Culture Media 98
4.4.2. Effect Of Medium pH On Bacterial Cell Production 98
4.4.3. Effect Of Growth Medium Modification With Chickweed Extracts 99
4.5. Literature Cited 99
CHAPTER 5 HOST RANGE 105
5.1. Abstract 105
5.2. Introduction 105
5.3. The Organisms 106
5.3.1. The Target Weeds: Common Chickweed 106
5.3.2. The Biocontrol Agent: *Burkholderia Andropogonis* 107
5.4. Methods and Materials 108
5.4.1. Inoculum Preparation 108
5.4.2. Plant Production 108
5.4.3. Treatments 109
5.4.4. Measurements 109
5.5 Results And Discussions 109
5.6. Literature Cited 111
CHAPTER 6 EFFICACY 121
6.1. Abstract 121
6.2. Introduction 121
6.3. Materials and Methods 121
6.3.1. Efficacy Under Greenhouse Conditions 121
6.3.1.1. Inoculum Preparation. 121
6.3.1.2. Plant Production. 122
6.3.1.3. Inoculation 123
6.3.1.4. Effect Of Bacterial Concentration On Disease Severity. 123
6.3.1.5. Effect Of Spray Solution pH On Disease Severity 123

6.3.1.6. Effect Of Surfactant Type And Concentration On Disease Severity. ..124
6.3.1.7. Effect Of Repeat Application On Disease Severity. 124
6.3.1.8. Effect Of Bacterial Growth Medium And Inoculum Growth Stage On .... Efficacy. 124
6.3.1.9. Effect Of Bacterial Growth Stage And Chickweed Growth Stage On ..... Disease Severity. 125
6.4.1.10. Infectivity And Disease Severity On Group 2 Herbicide Resistant ..... Chickweed. 125
6.3.2. Efficacy Under Field Conditions 126
6.3.2.1. Experiments Conducted In Outside Pots 126
6.3.2.2. Experiments Conducted In Field Plots. 127
6.4. Results and Discussions 128
6.4.1. Efficacy Under Greenhouse Conditions 128
6.4.1.1. Effect Of Bacterial Concentration On Disease Severity. 128
6.4.1.2. Effect Of Spray Solution pH. 128
6.4.1.3. Effect Of Surfactant Type and Concentration On Disease Severity. ...128
6.4.1.4. Effect Of Repeat Application On Disease Severity. 128
6.4.1.5. Effect Of Bacterial Growth Medium and Inoculum Growth Stage On ..... Disease Severity. 129
6.4.1.6. Effect Of Inoculum Growth Stage And Chickweed Growth Stage On ..... Disease Severity. 129
6.4.1.7. Infectivity And Disease Severity On Group 2 Herbicide Resistant ..... Chickweed. 130
6.4.2. Efficacy Under Field Conditions 130
6.4.2.1. Experiments Conducted In Outside Pots 130
6.4.2.2. Experiments Conducted In Field Plots. 130
6.5. Literature Cited 131
CHAPTER 7 NATURAL PRODUCTS 144
7.1 Abstract 144
7.2. Introduction 144
7.3 Materials and Methods 144
7.3.1. Heat Stability Of The Cell-Free Culture Filtrates 145
7.3.2. Effect Of The Cell-Free Culture Filtrates On Chickweed Root Growth 145
7.3.3. Diseases Caused By The Cell-Free Culture Filtrates 145
7.4. Results And Discussions 146

7.5. Literature Cited 146
DELIVERABLES 149

List of Tables

PART I - Cleavers Bioherbicide

PAGE

Table 2.1. Result of Analysis of Variance (ANOVA, SAS) for mycelial growth, spore production, and spore germination of Plectosporium tabacinum.....24

Table 2.2. Effect of culture media and light regime on mycelial growth and sporulation of *Plectosporium tabacinum**. 25

Table 2.3. Effect of temperature on spore germination of *Plectosporium tabacinum* 26

Table 2.4. Effect of liquid culture media on spore production and efficacy against false cleavers of *Plectosporium tabacinum**. 27

Table 2.5. Effect of nitrogen source on spore production and efficacy against false cleavers of *Plectosporium tabacinum* in a basal salts medium based on Richard's solution*.....28

Table 2.6. Effect of carbon source on spore production and efficacy against false cleavers of *Plectosporium tabacinum* in a basal salts medium based on Richard's solution*.....29

Table 2.7. Effect of carbon concentration on spore production and efficacy against false cleavers of *Plectosporium tabacinum* in a basal salts medium based on Richard's solution*.....30

Table 3.1. Test plant species used for host-specificity screening of *Plectosporium tabacinum* against false cleavers (*Galium spurium*) based on the modified centrifugal phylogenetic and varietal strategy 47

Table 3.2. Pathogenicity of *Plectosporium tabacinum* on herbicide-resistant and herbicide-susceptible false cleavers and various cultivars of canola*.....49

Table 3.3. Disease severity results of host specificity screening for *Plectosporium tabacinum*.....50

Table 4.1. Effect of repetitive dew periods on mortality and reduction in dry weight of *Galium spurium* inoculated with *Plectosporium tabacinum**.....61

PART II - Chickweed Bioherbicide

PAGE

Table 2.1 Number of fungi and bacteria isolated from chickweed at various locations 84

Table 2.2 Virulence of fungal isolates on chickweed (Stellaria media)* 85

Table 2.3 Virulence of bacterial isolates on chickweed (Stellaria media)* 86

Table 3.1 Carbon assimilation of CW00B006C as opposed to type strain of Burkholderia andropogonis* 94

Table 4.1 Comparison of exponential growth parameters of CW00B006C among four liquid culture media* 100

Table 5.1 Test plant species used for host-specificity screening of Burkholderia andropogonis against common chickweed (Stellaria media) based on the modified centrifugal phylogenetic and varietal strategy 115

Table 5.2 Disease severity results of host specificity screening for Burkholderia andropogonis 118

Table 5.3 Worldwide distribution, economic importance, and Canadian native flora and their habitats in the Order Caryophyllales[a] 119

List of Figures

PART I - Cleavers Bioherbicide

PAGE

Figure 2.1. Effect of the nutrient strength of agar media on the radial mycelial growth (A) and conidia production (B) of *Plectosporium tabacinum*. The number of conidia per plate was determined after 21 d incubation while mycelial growth was measured after 14 d incubation. Results of two trials with three replicates per trial were combined for each medium. Standard errors are indicated by vertical bars. 31

Figure 2.2. Effect of the pH of buffered and unbuffered potato dextrose agar media on the radial mycelial growth (A) and conidia production (B) of *Plectosporium tabacinum*. The number of conidia per plate was determined after 21 d incubation while mycelial growth was measured after 14 d incubation. Results of two trials with three replicates per trial were combined for each medium. Standard errors are indicated by vertical bars. 32

Figure 2.3. Effect of temperature on the radial mycelial growth (A) and conidia production (B) of *Plectosporium tabacinum* on potato dextrose agar (PDA), Czapek Dox agar (CDA), lima bean agar (LBA) and V-8 juice agar (VA). The number of conidia per plate was determined after 21 d incubation while mycelial growth was measured after 14 d incubation. Results of two trials with three replicates per trial were combined for each medium. Standard errors are indicated by vertical bars. 33

Figure 2.4. Effect of osmotic potential on spore production of *Plectosporium tabacinum*. The number of spores/ml was determined after 72 h incubation on an orbit shaker at 150 rpm. 34

Figure 2.5. Effect of initial spore concentration on spore production of *Plectosporium tabacinum* in Richard's solution. The number of spores/plate was determined after 3 days incubation on an orbit shaker at 150 rpm. 35

Figure 2.6. Spore production over incubation time of *Plectosporium tabacinum* in Richard's solution. The number of spores/plate was determined after 3 days incubation on an orbit shaker at 150 rpm. .36

Figure 2.7. Effect of carbon-to-nitrogen ratio on spore production of *Plectosporium tabacinum* in a basal salt medium of Richard's solution. Media with three carbon (sucrose) and nitrogen (KNO_3) concentrations and six different ratios of carbon to nitrogen (C:N) was prepared. Media with sucrose

concentrations of 8.4 (A), 21 (B), and 33.6 (C) g L^{-1} and C:N rations of 40:1, 20:1, 15:1, 10:1, 7.5:1, and 5:1 were prepared. Spore production in unmodified Richard's solution (50 g L^{-1} sucrose and a 5:1 C:N ratio) was included as a control. The number of spores/plate was determined after 3 days incubation on an orbit shaker at 150 rpm. 37

Figure 3.1. The disease reaction of false cleavers caused by the fungal pathogen *Plectosporium tabacinum*. (A) False cleavers at cotyledon stage and (B) False cleavers at 1-whorl stage. Left – Control and Right – Treated. 51

Figure 4.1. Effect of *Plectosporium tabacinum* inoculum concentration and plant growth stage on the control of false cleavers, expressed as percent mortality (A) and reduction in dry weight (B) seven days after inoculation. Seedlings of false cleavers at the cotyledon, and 1-, 2-, 3-, 4- or 5-whorl growth stage of false cleavers were inoculated with a conidial suspension at concentrations of 1 x 10^8, 1 x 10^7, 1 x 10^6, 1 x 10^5 or 0 spores/ml in 1% gelatin solution. After inoculation, pots with inoculated seedlings were placed in a dark dew chamber for 24 h. Data from two trials were not pooled because variances were not homogenous. Data represent the mean of three replicates. 62

Figure 4.2. Effect of dew period on disease development caused by *Plectosporium tabacinum* on false cleavers, expressed as percent mortality (A) and reduction in dry weight (B) seven days after inoculation. Seedlings of false cleavers at the 1-whorl growth stage were inoculated with a conidial suspension at a concentration of 1 x 10^7 conidia/ml in 1% gelatin solution. Dew temperature was 22 °C (in dark). Data from two trials were not pooled because variances were not homogenous. Data represent the mean of three replicates. 63

Figure 4.3. Effect of inoculum concentration and dew period duration on disease development caused by *Plectosporium tabacinum* on false cleavers, expressed as percent mortality (A) and reduction in dry weight (B) seven days after inoculation. False cleavers seedlings at the 1-whorl stage were inoculated with conidial suspension at a concentration of 5x10^6, 1x10^7, 5x10^7, or 1x10^8 conidia/ml in 1% gelatin solution. Dew temperature was 22 °C (in dark) and were placed in the dew chamber for 9, 12, 16, or 20 h and then returned to the greenhouse. Data from two trials were not pooled because variances were not homogenous. Data represent the mean of three replicates. 64

Figure 4.4. Effect of delaying initial dew period on disease development caused by *Plectosporium tabacinum* on false cleavers, expressed as percent mortality (A) and reduction in dry weight (B) seven days after inoculation. Seedlings of false cleavers at the 1-whorl growth stage were inoculated

with a conidial suspension at a concentration of 1 x 10^7 conidia/ml in 1% gelatin solution. Dew temperature was 22 °C (in dark). Data from two trials were not pooled because variances were not homogenous. Data represent the mean of three replicates. 65

Figure 5.1. Daily weather conditions recorded during the field evaluation of cleavers control with *Plectosporium tabacinum* spores. (A) Maximum and minimum temperatures (°C), (B) Precipitation (mm), (C) Sunlight period (h), and (D) Average radiation (watts/m^2)....70

Figure 5.2. Control of false cleavers (*Galium spurium*) with *Plectosporium tabacinum* spores under field conditions. A. Treatment with *P. tabacinum* spores (1 x 10^7 spores/ml) in oil emulsion formulation, and B. Oil emulsion formulation check without *P. tabacinum* spores (CK). The oil emulsion formulation consisted of a 10% oil phase and a 90% water phase. The oil phase consisted of unrefined corn oil. The water phase consisted the spore suspension, 0.5% macerated solidified PDA, and 0.1% Silwet®. Before spray, the two phases were mixed and blended at high speed in a blender for 45 s. False cleavers seedlings at the cotyledon, 1-whorl, or 2-whorl stage were treated with water (CK), the oil emulsion formulation without *P. tabacinum* spores (CK), or the oil emulsion formulation of *P. tabacinum* at a density of 5.0 x 10^8 spores/m^2....71

Figure 5.3. Daily weather conditions recorded during the field evaluation of cleavers control with *Plectosporium tabacinum* culture filtrates. (A) Maximum and minimum temperatures (°C), (B) Precipitation (mm), (C) Sunlight period (h), and (D) Average radiation (watts/m^2). 72

Figure 5.4. Control of false cleavers (*Galium spurium*) with *Plectosporium tabacinum* culture filtrates (containing no living cells) at different application volumes under field conditions. A. Fries medium check without *P. tabacinum* + 0.1% Silwet® at an application volume of 30 ml (CK), B. *P. tabacinum* culture filtrates + 0.1% Silwet® at an application volume of 30 ml, C. Fries Medium check without *P. tabacinum* + 0.1% Silwet® at an application volume of 40 ml (CK), and D. *P. tabacinum* culture filtrates + 0.1% Silwet® at an application volume of 40 ml....73

PART II - Chickweed Bioherbicide

PAGE

Figure 2.1 Disease symptoms caused by the bacterial isolate CW00B006C in chickweed. 87

Figure 4.1 CW00B006C bacterial cell growth curve in four different liquid culture media over 44 h period. KB - King's medium B, NBY - nutrient broth yeast extract, NGB - nutrient glucose broth, TSD - tryptic soy dextrose. 101

Figure 4.2 Exponential growth of CW00B006C bacterial cells in four different liquid culture media from 2 to 22 44 h. KB - King's medium B, NBY - nutrient broth yeast extract, NGB - nutrient glucose broth, TSD - tryptic soy dextrose. The best regression equation between cell numbers (N) and time (T) are: (a) For KB, $N = 6.968 + 0.105T$, $r^2 = 0.9868$, (b) For NBY, $N = 7.437 + 0.110T$, $r^2 = 0.9317$, (c) For NGB, $N = 7.407 + 0.104T$, $r^2 = 0.9762$, and (d) For TSD, $N = 7.372 + 0.099T$, $r^2 = 0.9873$ 102

Figure 4.3 CW00B006C bacterial cell growth curve in two different liquid culture media over 64 h period. NGB - nutrient glucose broth, TSD - tryptic soy dextrose. Data from the two trials of each experiment were pooled because the variances of trials were homogeneous according to Bartlett's test (Gomez & Gomez, 1984). Data points represent means of six replications. 103

Figure 4.4 Effect of chickweed extract on CW00B006C bacterial cell production. 104

Figure 6.1 Effect of CW00B006C bacterial concentration on the disease severity of chickweed. 132

Figure 6.2 Effect of surfactant type and concentration on disease severity of chickweed caused by Burkholderia andropogonis 133

Figure 6.3 Effect of repeat application on disease severity of chickweed caused by Burkholderia andropogonis 134

Figure 6.4 Effect of bacterial growth medium and inoculum growth stage on disease severity of chickweed caused by Burkholderia andropogonis 135

Figure 6.5 Effect of chickweed growth stage on disease severity of chickweed caused by Burkholderia andropogonis 136

Figure 6.6 Effect of bacterial growth stage and chickweed growth stage on disease severity of chickweed caused by Burkholderia andropogonis 137

Figure 6.7 Daily weather conditions from July 24 to September 14, 2001 recorded during the field evaluation of chickweed control with bacterial strain CW00B006C. (A) Maximum and minimum temperatures (°C), (B) Precipitation (mm), (C) Sunlight period (h), and (D) Average radiation (watts/m^2). 138

Figure 6.8 Disease severity on chickweed grown in outside pots caused by Burkholderia 139

Figure 6.9 Infectivity of Burkholderia andropogonis on chickweed under field conditions 140

Figure 6.10 Daily weather conditions from May 27 to July 29, 2002 recorded during the field evaluation of chickweed control with bacterial strain CW00B006C. (A) Maximum and minimum temperatures (°C), (B) Precipitation (mm), (C) Sunlight period (h), and (D) Average radiation (watts/m^2). 141

Figure 6.11 Disease severity and dry weight reduction of chickweed caused by Burkholderia andropogonis 142

Figure 6.12 Chickweed control with the bacterium Burkholderia andropogonis (A) Field trial conducted in 2002 and (B) Detailed weed control performance in one plot. 143

Figure 7.1 Effect of cell free culture filtrates of CW00B006C on root growth of chickweed seedlings. . 148

PART I - Cleavers Bioherbicide

Summary

A fungal pathogen (CL98-103) was isolated from naturally infected cleavers plants and evaluated as a bioherbicide for the control of cleavers. This fungal pathogen was identified as *Plectosporium tabacinum* (van Beyma) M. E. Palm, W. Gams et Nirenberg, an anamorph of *Plectoshphaerella cucumerina* (Lindf) W. Gams. This is the first report of *P. tabacinum* as a pathogen of *Galium* species. *P. tabacinum* was non-pathogenic to both herbicide tolerant and conventional cultivars of both Argentine canola and Polish canola. *P. tabacinum* killed cleavers seedlings when applied at a concentration of 1×10^7 conidia/ml and provided with 16 h dew. The efficacy of *P. tabacinum* on herbicide-resistant cleavers was identical to that on herbicide-susceptible cleavers. Further host range tests on 35 plant species in 26 genera and 12 families demonstrated that *P. tabacinum* could be safely used in canola for the control of cleavers in western Canada. This fungus may provide a novel approach to managing herbicide-resistant false cleavers.

This study characterized the mycelial growth, spore production, and spore germination of *P. tabacinum* on different standard agar media over a range of media pH and temperatures and under different light conditions; the effect of liquid culture conditions (i.e. media, osmotic potential, initial spore concentration, and culture time) on spore production and weed control efficacy of *P. tabacinum*; and the effect of carbon source, nitrogen source, carbon concentration, and carbon-to-nitrogen ratio on spore production, spore size, spore nuclear number, and weed control efficacy of *P. tabacinum*. The data obtained in this study are the first comprehensive description on the culture and spore production of *P. tabacinum*. The findings indicate that *P. tabacinum* can be easily grown and made to sporulate on standard agar media and in submerged liquid cultures, indicating spore production is not a limiting factor for developing *P. tabacinum* as a bioherbicide for the control of cleavers.

False cleavers mortality and dry weight reductions caused by *P. tabacinum* were also assessed under single or combined factors, including pathogen inoculum concentration, plant growth stage, dew period (duration, frequency, and timing), and dew period temperature, to determine the optimum conditions that can be taken advantage of and the limiting factors that should be avoid or overcome. The minimum inoculum concentration required to kill false cleavers seedlings was 1×10^7 conidia ml^{-1} at an application rate of 150 ml m^{-2}. False cleavers seedlings in the cotyledon or 1-whorl growth stage were the most susceptible. Increasing inoculum concentration increased weed control efficacy on older false cleavers seedlings. When an adequate dew period was provided, 100% mortality and dry weight reduction occurred. The minimum dew period to achieve 100% mortality was 16 h. Delaying the initiation of the dew period by 24 h significantly reduced disease development. Short, repetitive dew periods only improved false cleavers control to a limited extend and the length of initial dew is a critical factor influencing false cleavers mortality due to *P. tabacinum*. The optimal dew period temperature for disease development was above 15 °C.

Field efficacy of *P. tabacinum*, using either spores or culture filtrates as the active ingredient, for control of cleavers was assessed. Results demonstrated that both fungal spores and culture filtrates (containing no living cells) show promise for cleavers control under field conditions. With culture filtrates (containing no living cells), 80-90% cleavers control was observed under field conditions. Few or no phytotoxicity was observed with blank medium control. No damage was observed on wheat seedlings. Less than 5% leaf damage was observed on canola seedlings.

However, this damage was completely recovered three weeks after treatment. With the fungal spores, a spore concentration of 1×10^7 spores/ml in 0.1% Silwet®, 10% unrefined corn oil, and invert emulsion formulation caused 10%, 50%, and 100% disease severity, respectively. Proper formulations are required for the successful use of *P. tabacinum* spores for cleavers control under field conditions.

In summary, *P. tabacinum* can be viewed as a potential bioherbicide product. A Canadian provisionally patent has been applied for and issued (No. 2,325,215). Three manuscripts have been published in referred journals, i.e. *Weed Science*, *Canadian Journal of Botany*, and *Biocontrol Science and Technology*. Three key areas have been identified for further development of this fungus as a weed control product, i.e. Second year field trial, formulation development, and integration into current weed control system.

Chapter 1. Background Information

False cleavers (*Galium spurium*) and cleavers (*G. aparine*) are weeds of significant economic impact in western Canada, especially for the pedigreed canola seed industry. Under the Canada Seeds Act, no cleavers seed is allowed in pedigreed canola seed and thus, pedigreed seed of canola cannot be produced on land infested with cleavers. Unfortunately, the 1997 weed surveys in the prairie provinces of Canada revealed that cleavers populations dramatically increased in the past 10 years. Cleavers rose in relative abundance by 36 rankings in Alberta, 27 in Saskatchewan, and 19 in Manitoba (Thomas et al., 1998). Increasing cleavers infestation is threatening pedigreed canola seed industry. A "Cleavers Alert" has recently been issued by Alberta Agriculture.

Some chemical herbicides can provide satisfactory control of cleavers. However, herbicide resistance in cleavers has been reported in Alberta, involving multiple resistance to chemicals in different groups (Group-2 and –4 herbicides, which are the major herbicides recommended for its control) (Beckie et al., 1997; Hall et al., 1998). The resistant cleavers population is tolerant to one of the products for which herbicide-tolerant canola has been developed. Increasing herbicide use against this weed is likely to lead to the development of more cases of multiple resistance. Therefore, there is an urgent need to search for alternative strategies for controlling this weed.

A collaborative research program has been established between Agriculture and Agri-Food Canada, Saskatoon and Alberta Research Council, Vegreville to explore and develop pathogenic fungi as bioherbicides for control of cleavers, thus combating the herbicide resistance by introducing novel mode of action. Various pathogenic fungal pathogens have been isolated from cleavers. Initial laboratory studies have demonstrated that the fungal pathogen CL98-103, identified as *Plectosporium tabacinum* (van Beyma) M. E. Palm, W. Gams et Nirenberg, an anamorph of *Plectoshphaerella cucumerina* (Lindf) W. Gams, can kill cleavers without any infections on canola. Thus, this fungal pathogen has been chosen for further study to develop it as a bioherbicide for the control of cleavers. The specific objectives of this study were:

1. to develop techniques for spore production,
2. to delimitate the host specificity,
3. to determine the optimum conditions and limiting factors for maximum effectiveness, and
4. to quantify the field efficacy to assess the commercialization potential.

Literature Cited

1. Beckie, H.J., Legere, A., Thomas, A.G., Juras, L.T., and Devine, M.D. 1997. Nature and occurrence of herbicide-resistant wild oat and green foxtail across Saskatchewan ecoregions. Proceedings, 1997 ECW Meetings, Charlottetown, PEI.
2. Charudattan, R. 1991. The mycoherbicide approach with plant pathogens. Pages 24-57 *in* TeBeest, D.O. (ed.) *Microbial Control of Weeds*. Chapman & Hall, New York.
3. Greaves and MacQueen, 1992. Bioherbicides: their role in tomorrow's agriculture. Pages 295-306 *in Resistance: Achievements and developments in combating pesticide resistance*. Sci. Symp., Harpenden, England.
4. Hall, L.M, K.M. Stromme, G.P. Horsman, and M.D. Devine. 1998. Resistance to acetolactate synthase inhibitors and quinclorac in a biotype of false cleavers (*Galium spurium*). Weed Sci. 46:390-396.
5. Thomas, A.G., Frick, B.L., Juras, L.T, Hall, L., van Acker, R., and Joose, D. 1998. Changes in weed distributions indicated by quantitative surveys in the prairie provinces of Canada over 10 years. Proceedings of the 38th Annual WSSA Conference, Chicago, IL.

Chapter 2 Growth and Spore Production of *Plectosporium tabacinum*

2.1. Abstract

Plectosporium tabacinum has been evaluated as a bioherbicide for the control of false cleavers. Little is known, however, about the specific culture and sporulation requirements of *Plectosporium tabacinum* on standard agar media. In addition, no information on submerged liquid culture spore production of this fungus is available. These pieces of information are important for the successful culture and further development of this fungus. This study characterized the mycelial growth and sporulation of *Plectosporium tabacinum* on 14 different standard agar media over a range of light regimes, pH, and temperatures. Conditions required for submerged liquid culture spore production and resulting weed control efficacy of *Plectosporium tabacinum* were also assessed including the effect of liquid culture medium and pH and the effect of carbon-nitrogen such as carbon source, nitrogen source, carbon concentration, and carbon-to-nitrogen ratio. Results demonstrated that Potato dextrose agar (PDA) is the best agar medium for growth and spore production of *Plectosporium tabacinum*. On standard agar media, growth and spore production of *Plectosporium tabacinum* was not influenced by light regime. Medium pH did not affect mycelial growth and spore germination of *Plectosporium tabacinum*, but the optimal pH for spore production was 7. The optimal temperature for the mycelial growth was between 22 and 25 °C, but the optimal temperature for spore production was at either 20 or 30 °C, depending upon the nutrient medium. Temperatures above 20 °C were required to achieve greater than 90% spore germination. For submerged liquid culture spore production, Richard's solution (RS) is the best liquid medium for spore production of *Plectosporium tabacinum*. The optimal pH was 7 for RS but varied with culture media. Potassium nitrate and cornstarch were the best nitrogen source and carbon source, respectively. A medium with a carbon concentration of 12.6 g/L and a C:N ratio of 7.5:1 appears to be optimum for spore production and weed control efficacy of *Plectosporium tabacinum*.

2.2.Introduction

Plectosporium tabacinum (van Beyma) M. E. Palm, W. Gams et Nirenberg has been evaluated as a bioherbicide for the control of false cleavers (*Galium spurium* L.) in canola grown in western Canada (Zhang and Bailey 2000). The development of low-cost

methods for mass production of spores is an important step for the commercialization of a bioherbicide (Stowell et al. 1989; Boyette et al. 1991). Information available on factors influencing mycelial growth and spore production of *P. tabacinum* is scarce. Chung et al. (1998) reported that the optimum temperature for growth and sporulation of a *P. tabacinum* isolate was 25 to 30°C on four different agar media. Zazzerini & Tosi (1987) reported the optimum temperature for growth on agar media of another *P. tabacinum* isolate was 25 to 26°C. No comprehensive studies on the effects of nutritional conditions such as culture medium, and environmental conditions such as light regime, medium pH, and temperature, on growth and spore production of *P. tabacinum* have been reported. Such information is important for the successful culture of this fungus and would assist in selecting the best medium and the optimum conditions for its growth and spore production.

Submerged liquid culture spore production is the preferred technique for mass production of biocontrol agents because the technology is readily available and the scale-up process from the research phase to the development phase is relatively easy (Churchill 1982; Stowell et al. 1989; Boyette 1991). So far, no information has been reported on submerged liquid culture spore production for *P. tabacinum* (Palm et al. 1995; Chung et al. 1998; Smither-Kopperl et al. 1999). Factors such as medium type, pH, and carbon-nitrogen composition including nitrogen source, carbon source, carbon concentration, and carbon-to-nitrogen (C:N) ratio usually affect submerged spore production of fungal pathogens or biocontrol agents (Murray and Walter 1991; Jackson 1997). In particular, the carbon-nitrogen composition of the medium may affect not only the number of spores produced, but also spore characteristics and weed control efficacy (Jackson and Bothast 1990; Jackson and Schisler 1992; Jackson 1997). For instance, spores of *Colletotrichum truncatum* (Schw.) Andrus & Moore, a bioherbicide of hemp sesbania (*Sesbania exaltata* (Raf.) Rydb. ex A.W.Hill), produced in a medium with a C:N ratio of 10:1 were longer and thinner, more likely to be multinucleate, germinated more rapidly, formed more appressoria, and produced more disease in hemp sesbania than spores produced in media with a C:N ratio of either 30:1 or 80:1 (Jackson and Bothast 1990; Jackson and Schisler 1992). Although the genus *Plectosporium* is taxonomically similar to *Colletotrichum* (Palm et al. 1995), it is unknown whether carbon-nitrogen composition of the medium also affects its spore characteristics and weed control efficacy.

The objectives of this study were (1) to characterize the mycelial growth, sporulation, and spore germination of *P. tabacinum* on solid culture medium using different standard agar media over a range of light regimes, pH, and temperatures and (2) to assess the effect of liquid culture medium, pH, and carbon-nitrogen composition (including carbon source, nitrogen source, carbon concentration, and carbon-to-nitrogen ratio) on spore production, spore characteristics, and weed control efficacy of *P. tabacinum*.

2.3. Materials And Methods

2.3.1. General experimental procedure.

Stock cultures of single-spore *P. tabacinum* were stored in 15% glycerol at -80 °C. A cryovial of stock culture was warmed to room temperature in a 36°C water bath and the contents were dispensed in 200 µl aliquots on the surface of PDA Petri plates, then spread with a sterile glass rod. The Petri plate was incubated for 5 to 8 days at 22°C under 12 h cool white fluorescent light provided by two, 20W, 60 cm long tubes placed 30 cm above the Petri plates. Agar plugs with mycelium (5-mm diameter) from the margin of these young colonies were used as 'seed' inoculum for all experiments carried out on solid cultures. A spore solution of 1×10^7 spore/ml obtained by scraping Petri plates and suspending spores in sterile distilled water was used as 'seed' inoculum for all experiments conducted in submerged liquid cultures unless otherwise specified.

For the study on standard agar media, radial mycelial growth was determined after 14 d incubation using one colony diameter measurement per replicate Petri plate as growth of colonies was symmetrical. Total spore number per replicate Petri plate was determined after 21 d incubation. Spores were harvested by flooding Petri plates with 10 ml of distilled water and scraping the surface of the colonies with a glass slide. The resulting suspensions were agitated with a vortex mixer for 5 s and conidia from replicate Petri plates were counted individually with the aid of a haemacytometer. For spore germination tests, agar discs (16-mm diameter) were prepared and placed onto supporting slides. Droplets (10 µl) of spore suspension at a concentration of 1.0×10^5 spore/ml were placed on the discs and spread evenly on the surface using a spatula. The glass slides supporting the agar discs were placed into a humid chamber under ambient lab temperatures. After 24 h of incubation, 12.5 µl droplets of lactophenol-cotton blue were

placed on the agar discs to arrest further germination. Spores were considered to have germinated when the length of the germ-tube was greater than the width of the spore. Several randomly selected fields of view were examined using a compound microscope until a total of 100 spores per replicate had been assessed.

For submerged liquid culture spore production studies, a total of 200 ml of medium was placed in 500-ml Erlenmeyer flasks and pH was adjusted to 7.0 using 1N NaOH or HCl for all experiments unless otherwise specified. Each flask was inoculated with 1ml of 'seed' inoculum resulting in a spore concentration of 5×10^4 spores/ml in each flask at the start of each experiment. Inoculated flasks were incubated on an orbital shaker at 150 rpm under ambient laboratory conditions (24 ± 3°C). After 3 d incubation, spores were harvested by grinding the contents of each flask using an electric hand blender (Braun Multipractic MR 20, Lunnfield, MA, USA). The spores harvested from each flask were counted with the aid of a haemacytometer. The spore size was measured by preparing a wet mount slide of a 1×10^7 spores/ml solution and measuring the length and width of 10 random spores. To determine the number of nuclei per spore, a concentrated spore solution was prepared by filtering ground flask contents through 2-ply cheesecloth, placing the filtrate from each flask in a 250-mL Nalgene centrifuge bottle, and centrifuging solutions at 3300 rpm (2192 rcf) for 15 minutes at 23°C (Eppendorf 5810R, Brinkman Instruments Inc., Westbury, NY, USA). About 500 µl of each spore pellet was resuspended in 10 ml of sterile distilled water. The resulting spore suspension was fixed, cleared, and stained as described by Ward and Ciurysek (1961). The nuclear number of 10 randomly chosen spores was counted by examining prepared slides under a 100x oil immersion objective (Nikon Plan /1.25 oil, Ph3 DL) using a Nikon Eclipse E600 compound microscope with 10x eyepieces (Nikon Inc., Melville, NY, USA).

For testing weed control efficacy, seedlings of false cleavers were grown by placing 7-10 seeds in each 10 cm diameter peat pot containing steam-pasteurized soil mix. The soil mix consisted of 140 L soil, 107 L sand, 160 L Sunshine Growing Mix (SunGro Horticulture, Bellevue, Washington), 62 L Fibrous Blond Shagnum Peat Moss (Premier Pro Moss, Rivere-du-Loup, Quebec), 113 L vermiculite (Therm-O-Rock, Chandler, Arizona), 230 g dolomite lime, 150 g Super Phosphate (0-45-0) per batch of soil. After germination, seedlings were thinned to 5 plants per pot. Spores of *P. tabacinum* for efficacy testing were prepared by filtering and centrifuging ground flask

contents as previously described. Supernatant was decanted off and the spore pellet was resuspended in 1% gelatin solution (Difco, Detroit, MI, USA) at a concentration of 1 x 10^7 spores/ml. A 30 ml aliquot of spore suspension of each treatment was amended to contain 0.6 g of macerated PDA. False cleavers plants at the 1st whorl stage were sprayed with 10 ml per pot of the appropriate inoculum using an H-set airbrush (Paasche Airbrush Company, Harwood Heights, IL, USA) at a pressure of 100 kPa. Control plants were sprayed with 1% gelatin and macerated PDA. About 30 min after spraying, pots were placed in a dark dew chamber (Percival Lighted Dew Chamber, Boone, IW, USA) with 100% relative humidity at 22°C for 16 h. Pots were then returned to the greenhouse for the remainder of the experiment. Greenhouse conditions throughout the experiment were 24/20 ± 5°C day/night temperature with a 16 h photoperiod. Seven days after inoculation, dry weight of aboveground biomass was assessed. Dry weight was obtained by cutting aerial parts at soil level, drying in paper bags for 48 h at 70°C, and weighing. The dry weight data, expressed as percent reduction in biomass compared with biomass of uninoculated controls, were used as the measurement of weed control efficacy for all experiments.

All experiments were performed twice. A randomized complete block design with three replicates was used for all experiments. The data from spore counts were subjected to log (1 + x) transformation for zero values and log (x) for non-zero values; the percentage data were arcsine-transformed (Gomez and Gomez, 1984). The results from the two trials of each experiment were not pooled because the variances of trials were not homogeneous after the Bartlett's test (Gomez and Gomez, 1984). A similar trend, however, was observed between the trials, so only the results from one trial were presented. Treatment means were separated using Duncan's multi-range test. All analyses were conducted using SAS/STAT software for IBM-PC (SAS Institute Inc., 1987).

2.3.2. Mycelial growth, sporulation, and spore germination on standard agar media

2.3.2.1. Effect of culture media and light regime.

The experiment was a factorial experiment with 14 culture media and two light regimes. Culture media tested were water agar (WA), Cooke rose bengal agar (CRBA), dextrose tryptone agar (DTA), Czapek Dox agar (CDA), tryptic soy agar (TSA), malt extract agar (MEA), sabouroud dextrose agar (SDA), oatmeal agar (OMA), prune agar (PA), lima

bean agar (LBA), cornmeal agar (CA), V-8 juice agar (VA), and synthetic nutrient poor agar (SNA). All media were prepared according to the manufactures instructions using pre-prepared dehydrated media from Difco Laboratories (Detroit, MI, USA) or according to the methods described by Tuite (1969). Media were dispensed into plastic Petri plates (20 ml of medium/90-mm diameter plate) and were inoculated with a plug of 'seed' inoculum of *P. tabacinum*. Two light regimes, presence of light (12 h light/12 h dark) and absence of light (24 h dark), were included. Light conditions were created using cool white fluorescent light provided by two, 20W, 60 cm long tubes placed 30 cm above the Petri plates, while the absence of light was created by placing Petri plates in polyethylene boxes covered with aluminum foil so that both sets of plates could be grown in the same incubator (22 °C). Radial mycelial growth, sporulation, and spore germination rate were then determined.

2.3.2.2. Effect of PDA strength and light regime.

Since maximum sporulation occurred on PDA, radial mycelial growth, spore production, and spore germination of *P. tabacinum* were further evaluated under 7 different PDA strengths and two light regimes. Media with different concentrations of PDA were prepared by combining 1 L distilled water, 15 g L^{-1} Bacto-agar (Difco) and 24, 20, 16, 12, 8, 4 or 0 g L^{-1} potato dextrose broth (PDB; Difco) to prepare PDA, 5/6PDA, 2/3PDA, 1/2PDA, 1/3PDA, 1/6PDA, and WA respectively. Each replicate Petri plate was inoculated with 'seed' inoculum of *P. tabacinum*, sealed, and incubated at 22 °C under two light regimes as previously described. Radial mycelial growth, sporulation, and spore germination rate were then determined.

2.3.2.3. Effect of pH.

Experiments were performed on PDA prepared with and without a buffer solution to allow comparison of the effects of indirect changes in pH caused by the fungal isolate with the effects of constant but varied pH on growth and sporulation. Thus, PDA was prepared in a 0.1 N solution of dibasic potassium phosphate and in distilled water. The pH of the media was adjusted using 1 N HCl or NaOH to achieve pHs of 4, 5, 6, 7, and 8 prior to sterilization. Petri plates of each treatment were inoculated with 'seed' inoculum,

sealed and placed in an incubated at 22°C with 12 h light as previously described. Radial mycelial growth, spore production, and spore germination rate were then determined.

2.3.2.4. Effect of temperature.

The effect of temperature on mycelial growth, sporulation, and conidial germination of *P. tabacinum* was studied using PDA, CDA, LBA and V-A prepared as previously described. Petri plates of each medium were inoculated with 'seed' inoculum, sealed and placed in an incubator at 10, 15, 20, 22, 25, or 30°C with 12 h light. Radial mycelial growth, spore production, and spore germination rate were then determined.

2.3.3. Spore production in submerged liquid cultures

2.3.3.1. Effect of liquid culture media.

Liquid culture media tested included: Tryptic Soy broth (TSB), Czapek-Dox broth (CDB), Yeast Extract broth (YEB), Nutrient Broth (NB), Potato Dextrose broth (PDB), Malt Extract broth (MEB), Richard's solution (RS), modified Richard's solution (MRS)(Daniel et al., 1973; Wymore et al., 1988), *Colletotrichum truncatum* medium (CTM)(Jackson et al., 1996), V-8 juice medium (VA), and *Trichoderma* medium (TM) (Tabachnik, 1989). The pH of all culture media was adjusted to pH 7.

Three, 5 mm agar plugs from 'seed' inoculum were used to inoculate each replicate flask. After 3 d incubation, spore production in each medium was determined. Weed control efficacy was evaluated only for treatments of CDB, TM, RS, MRS, VA, and YEB because other media did not produce sufficient spore numbers to carry out the test.

2.3.3.2. Effect of osmotic potential.

The effect of osmotic potential was tested using PDB. The osmotic potential of the medium was adjusted to –0.2, -0.4, -0.6, -0.8, -1.0, –1.2, -1.4, -1.6, or –1.8 MPa using the osmotic adjuster PEG 8000 (Punja, 1993). Spore production was assessed after 3 d of incubation. Weed control efficacy was evaluated 7 d after inoculation.

2.3.3.3. Effect of initial spore concentration.

Richard's solution was used to evaluate the effect of initial spore concentration on spore production and weed control efficacy. Each replicate flask was inoculated with 10, 8, 6, 4, 2, or 1 ml of 1 x 10^7 conidia/ml 'seed' inoculum aseptically obtained from a Petri plate

or from Richard's solution to achieve initial spore concentrations in each flask of 5×10^5, 4×10^5, 3×10^5, 2×10^5, 1×10^5, and 5×10^4 conidia/ml. Treatments inoculated with 3 or 6, 5 mm agar plugs from7 d old spread plate cultures of *P. tabacinum* were also included. Spore production and weed control efficacy were then assessed.

2.3.3.4. Spore production over incubation time.

Richard's solution was used to evaluate the spore production of *P. tabacinum* over incubation time. All flasks were inoculated at the same time and spores were harvested after 24, 34, 38, 42, 46, 48, 58, 62, 66, 70, and 72 h incubation. Spore production and weed control efficacy were then assessed.

2.3.3.5. Effect of nitrogen source.

The effect of nitrogen source on spore production, spore size, spore nuclear number, and weed control efficacy was tested using RS in which the potassium nitrate was substituted for various other nitrogen sources. For all nitrogen source treatments, a carbon concentration of 21 g/L and a C:N ratio of 15:1 were maintained. Nitrogen sources included corn gluten meal (12.42 g L^{-1}), corn steep liquor (23.99 g L^{-1}), malt extract (25 g L^{-1}), casein (10.1 g L^{-1}), yeast extract (13.24 g L^{-1}), bovine serum albumin (9.06 g L^{-1}), cottonseed oil (77.12 g L^{-1}), casamino acids (14.04 g L^{-1}), sodium nitrate (8.51 g L^{-1}), aspargine (6.62 g L^{-1}), glutamic acid (14.74 g L^{-1}), urea (3 g L^{-1}), and ammonium sulfate (6.62 g L^{-1}). Richard's solution made with potassium nitrate (10 g L^{-1}) was included as a control. Spore production, spore size, spore nuclear number, and weed control efficacy were then determined.

2.3.3.6. Effect of carbon source.

The effect of carbon source on spore production, spore size, spore nuclear number, and weed control efficacy was tested using RS for which the sucrose was substituted for various other carbon compounds. For all carbon source treatments, a carbon concentration of 21 g/L and a C:N ratio of 15:1 were maintained. Carbon sources included glucose, fructose, galactose, glycerol, potassium acetate, acetic acid, cellulose, corn starch and citrate. Richard's solution made with sucrose was included as a control. Spore production, spore size, spore nuclear number, and weed control efficacy were then determined.

2.3.3.7. Effect of carbon concentration.

Richard's solution was used as the medium. To maintain a C:N ratio of 15:1, the carbon concentration of the medium was adjusted to 4.2, 8.4, 12.6, 16.8, 21.0, 25.2, 29.4, or 33.6 g/L by adding certain amount of sucrose and potassium nitrate. Spore production, spore size, spore nuclear number, and weed control efficacy were then determined.

2.3.3.8. Effect of carbon-to-nitrogen (CN) ratio.

For the C:N ratio studies, RS was used as the medium. Media with sucrose concentrations of 8.4, 21.0, and 33.6 g L^{-1} and C:N ratios of 40:1, 20:1, 15:1, 10:1, 7.5:1, and 5:1 were prepared. Spore production in Richard's solution (50 g L^{-1} sucrose and a 5:1 C:N ratio) was included as a control. Spore production, spore size, spore nuclear number, and weed control efficacy were then determined.

2.4. Results

2.4.1. Mycelial growth, sporulation, spore germination on standard agar media

2.4.1.1. Effect of culture media and light regime.

According ANOVA, culture media significantly affect the radial mycelial growth and sporulation but did not impact spore germination of *P. tabacinum* (Table 2.1). Similarly, light regime did not influence the radial growth, sporulation, and spore germination of *P. tabacinum*. An interaction between light regime and culture media existed only for mycelial growth but not for sporulation and spore germination.

The maximum radial mycelial growth occurred on VA, SNA, and CA (Table 2.2). The greatest numbers of spores was produced on PDA, followed by VA (Table 2.2). On some media, mycelial growth of *P. tabacinum* was influenced by light conditions. For example, mycelial growth was significantly greater on CRBA under dark conditions than under 12 h light/12 h dark. A 100% spore germination rate was observed for all light regime and culture media treatments.

2.4.1.2. Effect of PDA strength and light regime.

PDA strengths did not affect mycelial growth and spore germination but significantly influenced sporulation of *P. tabacinum* (Figure 2.1). Sporulation of *P. tabacinum* increased with increasing PDA strengths. There was no effect of light regime and

interaction between light regimes and PDA strengths on mycelial growth, sporulation, and spore germination. One hundred percent of spores germinated on all media having different nutrient content.

2.4.1.3. Effect of pH.

Medium pH did not affect the radial mycelial growth of *P. tabacinum*, either with or without phosphate buffer (Figure 2.2A). However, sporulation of *P. tabacinum* was significantly influenced by medium pH under both buffered and unbuffered conditions (Figure 2.2B). When PDA was prepared without phosphate buffer, sporulation increased with an increase of pH, to maximum at pH 7, and decreased at pH 8. The presence of buffer in PDA reduced the number of spores produced by *P. tabacinum* at all pHs (Figure 2.2B). The amount of reduction in sporulation was dependent on the pH of the medium and this accounts for the interaction between pH and buffer. The pH of the media did not affect spore germination regardless of treatment. One hundred percent germination rate was observed.

2.4.1.4. Effect of Temperature.

The radial mycelial growth on all media steadily increased as the incubation temperature was increased from 10°C to 22°C or 25°C (Figure 2.3). *P. tabacinum* grew comparatively better at the higher temperature (20-30°C) than at 10°C and 15°C. Temperatures of both 20°C and 30°C induced greater sporulation than at 10, 15, or 25°C. The VA medium proved to be an exception with maximum sporulation at 20°C followed by a steady decline in sporulation as the incubation temperature was increased. Sporulation was highest at 20°C on all media except PDA, for which sporulation reached a maximum at 30°C. Spore production at 10°C and 15°C was comparatively low on all media. An interaction between medium and temperature existed for both the radial mycelial growth and spore production.

Temperatures significantly affected spore germination of *P. tabacinum* (Table 2.3). Increasing temperature between 10 and 30 °C increased spore germination. Over 90% spores germinated when temperatures were above 20 °C, while less than 10% of spores germinated when temperatures were below 15 °C.

2.4.2. Spore production in submerged liquid cultures

2.4.2.1. Effect of liquid culture media.

Spore production of *P. tabacinum* varied in different liquid culture media (Table 2.4). The most abundant sporulation occurred in VM, in which 2.62 x 10^7 spores/ml were produced within 3 days. Two other media, RS and YEB, produced more than 1 x 10^7 spores/ml, but all other media produced less than 10^7 spores/ml. The greatest reduction in dry weight of false cleavers was observed with spores produced in TM (Table 2.4). Similar reductions in dry weight were observed with spores produced from RS (97.1% dry weight reduction), VM (84%) and YEB (79%). RS was chosen as the base medium for further characterization of spore production of *P. tabacinum* in submerged culture because it had high spore production, high efficacy, and was a simple defined medium.

2.4.2.2. Effect of osmotic potential.

Spore production of *P. tabacinum* increased with a reduction in osmotic potential from –0.2 to –0.6 MPa (Figure 2.4). Further decreases in osmotic potential reduced spore production where below on osmotic potential of –1.2 MPa, spore production was negligible.

2.4.2.3. Effect of initial spore concentration.

Spore production of *P. tabacinum* increased with an increasing initial spore concentration (Figure 2.5). Within 3 days, highest spore numbers (4.25 x 10^7 spores/ml) were obtained with the highest initial spore concentration (5 x 10^5 spore/ml), however, there was no significant difference in spore numbers among initial spore concentrations at 3 x 10^5 spore/ml, 4 x 10^5 spore/ml, and 5 x 10^5 spore/ml. With the lowest initial spore concentration at 5 x 10^4 spore/ml, spore production within 3 days still reached 1.6 x 10^7 spore/ml.

2.4.2.4. Spore production over incubation time.

No spores were produced before 34 h incubation. The exponentional spore production was observed from 38 h to 66 h after inoculation and incubation (Figure 2.6). Spore production of *P. tabacinum* did not increase significantly after 66 h of incubation indicating growth had reached the stationary phase.

2.4.2.5.Effect of nitrogen source.

Medium with corn gluten meal as nitrogen source produced significantly more spores than standard RS with potassium nitrate as the nitrogen source (Table 2.5). Media with nitrogen sources of asparagines, yeast extract, sodium nitrate, and casamino acids produced spore numbers similar to standard RS. All other nitrogen sources produced significant fewer spores than standard RS. Medium with cottonseed oil as the nitrogen source did not support growth and sporulation of *P. tabacinum*.

Nitrogen source did not influence the spore size and nuclear number of *P. tabacinum* but did affect the weed control efficacy of *P. tabacinum* on false cleavers (Table 2.5). Spores produced in media with corn steep liquor, casamino acids, potassium nitrate or yeast extract as the nitrogen source caused 90% - 100% dry weight reduction of false cleavers seedlings, while spores from media with other nitrogen sources resulted in only 50%-81% dry weight reduction of false cleavers seedlings.

2.4.2.6. Effect of carbon source.

Galactose or corn starch as carbon source produced significantly more spores (3.68-3.81 x 10^7 spores/ml) than the control medium with sucrose (2.45 x 10^7 spores/ml) (Table 2.6). The number of spores/ml produced with cellulose was similar to sucrose, but not significantly different from the poorer carbon sources of glucose, fructose, and citrate acid. Spore production was negligible with glycerol, potassium acetate, and acetate acid. Nuclear number of spores harvested from different carbon source treatments varied from 1.4 to 1.9 but statistically were not different (Table 2.6). Spore length and width, however, differed depending on the carbon source (Table 2.6). Sucrose, galactose, and citrate acid produced the longest spores while corn starch, cellulose, and glucose produced the shortest spores. Fructose produced spores of intermediate length. The widest spores were produced in a medium with a carbon source of galactose and the narrowest spores in a medium with cellulose as the carbon source. Spores produced in media with all other carbon sources had an intermediate width. Carbon source also influenced weed control efficacy of *P. tabacinum* on false cleavers (Table 2.6). Spores produced in a medium with carbon sources of sucrose, citrate acid, fructose, or corn starch caused more than 94% dry weight reduction of false cleavers seedlings, while

spores produced in media with other carbon sources resulted in less than 65% dry weight reduction of false cleavers seedlings.

2.4.2.7. Effect of carbon concentration.

When a C:N ratio of 15:1 was maintained, spore production of *P. tabacinum* was significantly influenced by carbon concentration (Table 2.7). Carbon concentrations between 8.4 and 16.8 g/L resulted in the highest levels of spore production (5.68-6.77 x 10^7 spores/ml). Carbon concentration of media with a C:N ratio of 15:1 did not influence the spore size and nuclear numbers. Spores from media of all carbon concentrations, except for 4.2 g/L, resulted in over 80% dry weight reduction of false cleavers seedlings. Spores from media with a carbon concentration of 4.2 g/L caused a dry weight reduction of false cleavers of less than 60%.

2.4.2.8. Effect of carbon-to-nitrogen (C:N) ratio.

No interactions were observed between carbon concentration and C:N ratio ($P > 0.1160$). With a carbon concentration of 8 g/L, media with C:N ratios of 5:1, 7.5:1, 10:1 produced a similar number of spores and significantly more spores than media with C:N ratios of 15:1, 20:1, and 40:1 (Figure 2.7). With a carbon concentration of 21 g/L, media with C:N ratios of 5:1, 7.5:1, 20:1, and 40:1 produced a similar number of spores and significantly more spores than media with C:N ratios of 10:1 and 15:1. With a carbon concentration of 33 g/L, spore production in medium with a C:N ratio of 7.5:1 was greatest. Spore production in this medium was significantly greater than that in medium with a C:N ratio of 15:1 but was similar to media with C:N ratios of 5:1, 10:1, 20:1, and 40:1.

A comparison of spore production for cultures grown with various carbon concentrations and the same C:N ratio demonstrated that media with all three carbon concentration of 8, 21, and 33g/L produced similar spore numbers. This trend was in accordance with that in the carbon concentration experiments with a C:N ratio of 15:1 where the spore production was not significantly different among media with carbon concentrations of 8, 21, and 33g/L. Overall, for the three carbon concentrations studied, a C:N ratio of 7.5:1 allowed the highest spore production.

No significant difference in weed control efficacy, spore size, and nuclear number was observed among treatments with different carbon concentrations and C:N ratios.

2.5. Discussion

Under laboratory conditions, radial mycelial growth, sporulation, and spore germination of *P. tabacinum* changed with changes in nutritional and environmental conditions, although light regime was not an important factor. Conidium production of *P. tabacinum* was most sensitive to changes in culture media, nutrient content of media, pH, and temperature. Radial mycelial growth was affected by culture media and temperature, but not by nutrient content of media and pH. Spore germination was only influenced by temperature and not by other factors studied.

The most abundant spores were produced on PDA, at about 10^9 spores per Petri plate. As the concentration of nutrients in the agar medium was decreased by decreasing the concentration of PDA, sporulation decreased. The optimum pH for sporulation of this fungus is around 7, while the optimum temperature for sporulation is between 20 and 30°C. This is in consistency with other findings (Zazzerini & Tosi, 1987; Chung *et al.*, 1998).

Since the optimum temperature for spore germination of this fungus was above 20°C, temperature may be a limiting factor for this fungus as bioherbicide used in Canada. One way to minimize the impact of this limitation is to apply this fungal pathogen in the daytime instead of late afternoon as suggested for most potential bioherbicides.

Palm et al. (1995) believe that *P. tabacinum* was taxonomically close to *Colletotrichum*. Spore germination *Colletotrichum* species is often decreased when spore concentration is increased (Lax et al., 1985; Morin et al., 1994; Tsurushima et al., 1995). In bioherbicide research and application, high inoculum densities are generally sprayed onto target weeds (Klein and Auld, 1995). Thus, self-inhibition of spore germination may cause some concerns. However, this self-inhibition of spore germination was not observed with *P. tabacinum* on agar media or the leaf surface, even when the spore concentration reached 5×10^8 spores/ml.

Culture media significantly influenced submerged spore production of *P. tabacinum*. In addition to influencing spore production, medium composition affected the weed control efficacy of *P. tabacinum* on false cleavers seedlings. Findings in this study suggested that Richard's solution is a good basal medium for spore production of *P.*

tabacinum because it produces high spore numbers with good weed control efficacy. A similar response was observed in other bioherbicide candidates (Jackson and Bothast, 1990; Jackson and Schisler, 1992; Jackson, 1997).

The studies on the effect of osmotic potential on sporulation of *P. tabacinum* indicated that the fungus is sensitive to osmotic potentials below -0.6 MPa. No growth and sporulation occurred below -1.2 MPa. Thus, *P. tabacinum* belongs to a fungal group that is intolerant of water stress (Griffin, 1981). At slightly reduced potentials, however, spore production was increased. A similar response to slight osmotic tensions was observed in other fungi (Adebayo et al., 1971; Cook et al., 1972; Punja, 1993). The sensitivity of *P. tabacinum* to osmotic stress may be an important consideration when formulating this fungus as a bioherbicide product.

P. tabacinum produces large quantities of spores in liquid fermentation within 3 d when the initial spore concentration is at 5×10^4 spore/ml. Thus, spore production is not a limiting factor for the development of this fungus as a bioherbicide for control of false cleavers as well as other target weeds such as arrowhead and hydrilla (Chung et al., 1998; Smither-Kopperl, 1998).

Under the conditions of these experiments, nutritional factors such as nitrogen sources, carbon sources, carbon concentrations, and C:N ratios significantly affected spore production of *P. tabacinum*. This is in agreement with the studies on *C. truncatum* (Jackson and Bothast, 1990; Jackson and Schisler, 1992; Jackson, 1997). However, the responses of *P. tabacinum* to nutritional factors were different from those of *Colletotrichum* spp. even though there is taxonomic similarity between these two genera (Palm et al., 1995). Studies with *C. truncatum* showed that nitrogen sources containing protein or protein hydrolysates produced significantly more spores than inorganic nitrogen sources. However, for *P. tabacinum*, inorganic nitrogen sources such as KNO_3 also gave high levels of sporulation. Moreover, the high spore production with KNO_3 was associated with high weed control efficacy, while nitrogen sources such as corn gluten meal and corn steep liquor had lower weed control efficacy in spite of high spore production. The addition of casamino acids enhanced spore production of *C. truncatum*, but failed to do so for *P. tabacinum*. Similarly, a carbon source of sucrose produced moderate spore numbers but caused the greatest weed control efficacy. This further supported our selection of Richard's solution as a basal medium for spore production of

P. tabacinum. As for *C. truncatum*, for any given C:N ratio, too low or too high a carbon concentration inhibited spore production of *P. tabacinum*. It appeared that the optimum carbon concentration for both spore production and weed control efficacy was between 8.4 and 16.8 g/L.

Jackson and Schisler (1992) reported that nutritional environment altered spore size, lipid and protein content, and weed control efficacy of *C. truncatum*. Finding in this study demonstrated that spore attributes of *P. tabacinum* were only slightly affected by nutritional factors. Weed control efficacy, however, was affected by nutritional factors. In conclusion, *P. tabacinum* can be easily grown and made to sporulate in both standard agar media and submerged cultures. Thus, it is a good bioherbicide candidate.

2.6. Literature cited

1. Adebayo, A.A. and Harris, R.F. 1971. Fungal growth responses to osmotic as compared to matric water potential. Soil. Sci. Soc. Am. Proc. 35:465-469.
2. Alderman, D.J. and Polglase, J.L. 1985. *Fusarium tabacinum* as a gill parasite in the crayfish, *Austropotamobius pallipes*. J. Fish Dis. 8:249-252.
3. Boyette, C.D., Quimby, P.C. Jr., Connick, W.J., Daigle, D.J. and Fulgham, F.E. 1991. Progress in the production, formulation, and application of mycoherbicide. Pages 209-222 *in* TeBeest, D.O. (ed) Microbial Control of Weeds. Chapman & Hall, New York.
4. Cooke, R.J., Papendick, R.I. and Griffin, D.M. 1972. Growth of two root-rot fungi as affected by osmotic and matric water potentials. Soil. Sci. Soc. Am. Proc. 36:78-82.
5. Chung, Y.R., Koo, S.J., Kim, H.T. and Cho, K.Y. 1998. Potential of an indigenous fungus, *Plectosporium tabacinum*, as a mycoherbicide for control of arrowhead (*Sagittaria trifolia*). Plant Dis. 82: 657-660.
6. Churchill, B.W. 1982. Mass production of microorganisms for biological control. Pages 139-156 *in* Charudattan, R. and Walker, H.L. (eds) Biological Control of Weeds with Plant Pathogens. John Wiley & Sons, New York.
7. Gomez, K.A. and Gomez, A.A. 1994. Statistical Procedures for Agricultural Research. 2nd Edition. John Wiley & Sons, Inc. New York.
8. Griffin, D.M. 1981. Water and microbial stress. Adv. Microb. Ecol. 5:91-136.

9. Hall, R.A. and Papierok, B. 1982. Fungi as biological control agents of arthropods of agricultural and medical importance. Parasitology 84:205-240.

10. Jackson, M.A. 1997. Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. J. Ind. Microbiol. Biotech. 19:180-187.

11. Jackson, M.A. and Bothast, R.J. 1990. Carbon concentration and carbon-to-nitrogen ratio influence submerged culture conidiation by the potential bioherbicide *Colletotrichum truncatum* NRRL 13737. Appl. Environ. Microbiol. 56:3435-3438.

12. Jackson, M.A. and Schisler, D.A. The composition and attributes of *Colletotrichum truncatum* spores are altered by the nutritional environment. Applied and Environmental Microbiol. 58:2260-2265.

13. Klein, T.A. and B.A. Auld. 1995. Influence of spore dose and water volume on a mycoherbicide's efficacy in field trials. Biol. Cont. 5:173-178.

14. Lax, A.R., Templeton, G.E. and Meyer, W.L. 1985. Isolation, purification, and biological activity of a self-inhibitor from conidia of *Colletotrichum gloeosporioides*. Phytopathol. 75:386-390.

15. Morin, L., J.L. Derby and E.G. Kokko. 1994. Infection process of *Colletotrichum gloeosporioides* f. sp. *malvae* on Malvaceae weeds. Mycol. Res. 100:165-172.

16. Murray, T.D. and Walter, C.C. 1991, Influence of pH and matric potential on sporulation of *Cephalosporium gramineum*. Phytopathol. 81:79-84.

17. Palm, M.E., Gams, W. and Nirenberg, H.I. 1995. *Plectosporium*, a new genus for *Fusarium tabacinum*, the anamorph of *Plectosphaerella cucumerina*. Mycologia. 87: 397-406.

18. Punja, Z.K. 1993. Influence of culture conditions on mycelial growth and phialospore production and germination in *Chalara elegans*. Can. J. Bot. 71:447-456.

19. Saad, A.T. and Black, L.L. 1981. A new fungus disease of cucurbits. (Abstr.) Phytopathology. 71: 902.

20. SAS Institute Inc. 1987. SAS/STAT Guide for personal computers. Version 6. SAS Institute Inc., Cary, NC.

21. Smither-Kopperl, M.L., Charudattan, R. and Berger, R.D. 1999. *Plectosporium tabacinum*, a pathogen of the invasive aquatic weed Hydrilla verticillata in Florida. Plant Dis. 83: 24-28.

22. Stowell, L.J., Nette, K., Heath, B. and Shutter, R. 1989. Fermentation alternatives for commercial production of a mycoherbicide. Pages 219-227 *in* Demain, A.L., Somkuti, G.A., Hunter-Cevera, J.C. and Rossmoore, H.W. (eds) Novel Microbial Products for Medicine and Agriculture. Society for Industrial Microbiology.

23. Tsurushima T, T. Ueno, H .Fukami, H. Irie, and M. Inoue. 1995. Germination self-inhibitor from *Colletotrichum gloeosporioides* f. sp. *jussiaea*. Molecular Plant-Microbe Interactions 8:652-657.

24. Ward, E. W. B. and Ciurysek, K. W. 1961. Somatic mitosis in a Basidiomycete. Can. J. Botany. 39: 1497-1503.

25. Zazzerini, A. and L. Tosi. 1987. New sunflower disease caused by *Fusarium tabacinum*. Plant Dis. 71: 1043-1044.

26. Zhang, W. and Bailey, K. L. 1999. Biological control of cleavers (Galium spurium and G. aparine) with pathogenic fungi: exploration and discovery, in *Proceedings of the X International Symposium on Biological control of Weeds* (Spencer N.R. eds), Bozeman, MT, pp95-101.

Table 2.1. Result of Analysis of Variance (ANOVA, SAS) for mycelial growth, spore production, and spore germination of Plectosporium tabacinum

Source	DF	Mycelial growth		Spore production		Spore germination	
		F Value	Pr > F	*F Value*	*Pr > F*	*F Value*	*Pr > F*
Model	37	80.93	0.0001	27.17	0.0001	0.53	0.7878
Culture media	13	217.43	0.0001	75.78	0.0001	0.02	0.9899
Light regime	1	0.14	0.7071	0.07	0.7939	0.03	0.9532
Culture media x Light regime	13	11.66	0.0001	0.33	0.9868	0.05	0.8932

Table 2.2. Effect of culture media and light regime on mycelial growth and sporulation of *Plectosporium tabacinum*[*]

	Colony diameter (mm)		Number of spores/plate (x 10^8)	
Medium	Light	Dark	Light	Dark
Potato dextrose agar	63.3 cde[**]	54.2 d	6.942 a	6.458 a
V-8 juice agar	76.0 a	71.8 a	2.113 b	1.653 b
Czapek-Dox agar	66.3 cd	67.5 b	1.046 c	0.933 b
Oatmeal agar	62.7 e	61.8 c	0.818 c	0.941 b
Lima bean agar	72.3 b	69.8 ab	0.588 cd	0.535 b
Tryptic soy agar	54.8 f	57.4 d	0.561 cd	0.208 b
Dextrose Tryptone agar	54.2 f	55.7 d	0.099 d	0.159 b
Cooke rose bengal agar	22.8 h	40.2 e	0.028 d	0.092 b
Prune agar	67.7 c	63.5 c	0.021 d	0.014 b
Malt extract agar	57.3 f	56.2 d	0.008 d	0.008 b
Synthetic nutrient-poor agar	74.3 ab	72.8 a	0.006 d	0.007 b
Sabouraud dextrose agar	38.8 g	39.0 e	0.006 d	0.006 b
Water agar	64.5 cde	62.7 c	0.005 d	0.003 b
Cornmeal agar	72.7 b	72.7 a	0.004 d	0.006 b

[*] Mycelial growth was measured after 14 days of incubation. The number of spores/plate was determined after 21 days of incubation.

[**] Values in each column sharing the same letter are not significantly different according to Duncan's multiple-range test ($\alpha \leq 0.05$).

Table 2.3. Effect of temperature on spore germination of *Plectosporium tabacinum*

Temperature (°C)	Germination (%)
10	1.5 d*
15	8.5 c
20	93.0 b
25	99.6 a
30	100.0 a

* Values in each column sharing the same letter are not significantly different according to Duncan's multiple-range test ($\alpha \leq 0.05$).

Table 2.4. Effect of liquid culture media on spore production and efficacy against false cleavers of *Plectosporium tabacinum*[*]

Medium	Spore production (x 10^7 spores/ml)	Percent dry weight reduction (%)
V-8 juice medium (VM)	2.62 a[**]	84.0 ab[**]
Yeast extract broth (YEB)	1.43 b	79.4 bc
Richard's solution (RS)	1.06 c	97.1 ab
Czapek-Dox broth (CDB)	0.49 d	62.1 c
Trichoderma medium (TM)	0.31 de	100.0 a
Tryptic soy broth (TSB)	0.30 de	-
Potato dextrose broth (PDB)	0.16 e	-
Modified Richard's solution (MSR)	0.14 e	-
Nutrient broth (NB)	0.05 e	-
Colletotrichum truncatum medium (CTM)	0.04 e	-
Malt extract broth (MEB)	0.02 e	-

[*] The spores were harvested after 72 h incubation.

[**] Values in each column sharing the same letter are not significantly different according to Duncan's multiple-range test ($\alpha \leq 0.05$). – denotes no test was done.

Table 2.5. Effect of nitrogen source on spore production and efficacy against false cleavers of *Plectosporium tabacinum* in a basal salts medium based on Richard's solution*

Nitrogen sources	Spore production (x 10^7 spores/ml)	Nuclear number	Spore length (μm)	Spore width (μm)	Percent dry weight reduction (%)
corn gluten meal	6.63 a**	1.6 a	7.4 a	3.2 a	80.8 ab
corn steep liquor	2.70 b	1.7 a	7.1 a	3.0 a	91.7 a
potassium nitrate	2.45 bc	1.8 a	7.4 a	3.4 a	100.0 a
glutamic acid	1.72 bcd	1.9 a	7.4 a	3.3 a	52.9 c
asparagines	1.67 bcd	1.6 a	7.0 a	3.0 a	50.7 c
casamino acids	1.54 bcd	1.8 a	7.6 a	3.1 a	93.5 a
yeast extract	1.54 bcd	1.8 a	7.5 a	3.4 a	100.0 a
sodium nitrate	1.48 bcd	1.9 a	7.3 a	3.2 a	65.6 bc
casein	0.83 bcd	-	-	-	-
urea	0.66 bcd	-	-	-	-
malt extract	0.20 cd	-	-	-	-
ammonium sulfate	0.13 cd	-	-	-	-
bovine serum albumin	0.12 cd	-	-	-	-
cottonseed oil	0.00 d	-	-	-	-

* For all nitrogen source treatments, a carbon concentration of 21 g/L and a C:N ratio of 15:1 was maintained in the basal salt medium. The spores were harvested after 72 h incubation.

** Values in each column sharing the same letter are not significantly different according to Duncan's multiple-range test ($\alpha \leq 0.05$).

Table 2.6. Effect of carbon source on spore production and efficacy against false cleavers of *Plectosporium tabacinum* in a basal salts medium based on Richard's solution*

Carbon sources	Spore production (x 10^7 spores/ml)	Nuclear number	Spore length (μm)	Spore width (μm)	Percent dry weight reduction (%)
galactose	3.81 a**	1.7	7.6 a	4.4 a	62.4 b
corn starch	3.68 a	1.8	6.3 b	3.4 c	94.6 a
sucrose	2.45 b	1.9	8.3 a	3.9 b	99.8 a
cellulose	1.62 bc	1.5	6.9 b	3.0 d	46.0 c
glucose	0.09 cd	1.4	6.9 b	3.9 b	63.5 b
fructose	0.08 cd	1.4	7.3 ab	3.9 b	96.0 a
citrate acid	0.06 cd	1.7	8.1 a	3.7 b	98.9 a
glycerol	0.004 d	-	-	-	-
potassium acetate	0.002 d	-	-	-	-
acetate acid	0.000 d	-	-	-	-

* For all carbon source treatments, a carbon concentration of 21 g/L and a C:N ratio of 15:1 were maintained in the basal salt medium. The spores were harvested after 72 h incubation.

** Values in each column sharing the same letter are not significantly different according to Duncan's multiple-range test ($\alpha \leq 0.05$).

Table 2.7. Effect of carbon concentration on spore production and efficacy against false cleavers of *Plectosporium tabacinum* in a basal salts medium based on Richard's solution*

Carbon concentration (g/L)	Spore production (x 10^7 spores/ml)	Nuclear number	Spore length (μm)	Spore width (μm)	Percent dry weight reduction (%)
4.2	5.23 bcd**	1.6 a	7.2 a	3.0 a	58.8 b
8.4	6.25 ab	1.7 a	7.6 a	3.2 a	81.9 a
12.6	6.77 a	1.7 a	7.5 a	3.1 a	90.0 a
16.8	5.68 abc	1.9 a	7.6 a	3.2 a	88.3 a
21.0	5.08 bcd	1.7 a	7.2 a	3.3 a	83.5 a
25.2	4.65 cde	1.7 a	7.4 a	3.0 a	90.8 a
29.4	4.37 de	1.7 a	7.0 a	3.3 a	83.4 a
33.6	3.68 e	1.8 a	7.0 a	3.1 a	81.9 a

* While maintaining a C:N ratio of 15:1, the carbon concentration of the basal salt medium (Richard's solution) was adjusted to 4.2, 8.4, 12.6, 16.8, 21.0, 25.2, 29.4, or 33.6 g/L by adding variable amount of sucrose and potassium nitrate. The spores was harvested and counted after 72 h incubation on a rotary shaker at 150 rpm.

** Values in each column sharing the same letter are not significantly different according to Duncan's multiple-range test ($\alpha \leq 0.05$).

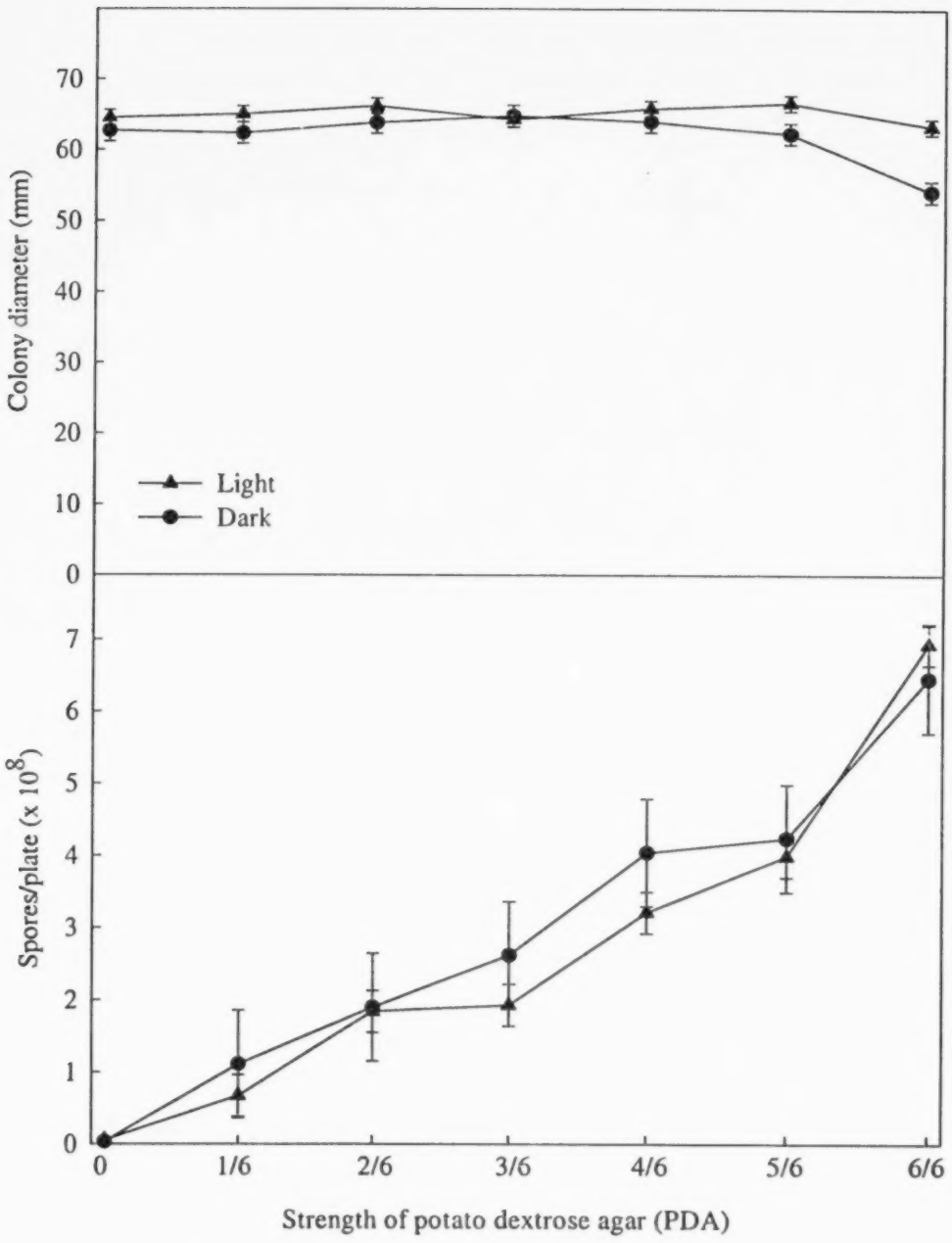

Figure 2.1. Effect of the nutrient strength of agar media on the radial mycelial growth (A) and conidia production (B) of *Plectosporium tabacinum*. The number of conidia per plate was determined after 21 d incubation while mycelial growth was measured after 14 d incubation. Results of two trials with three replicates per trial were combined for each medium. Standard errors are indicated by vertical bars.

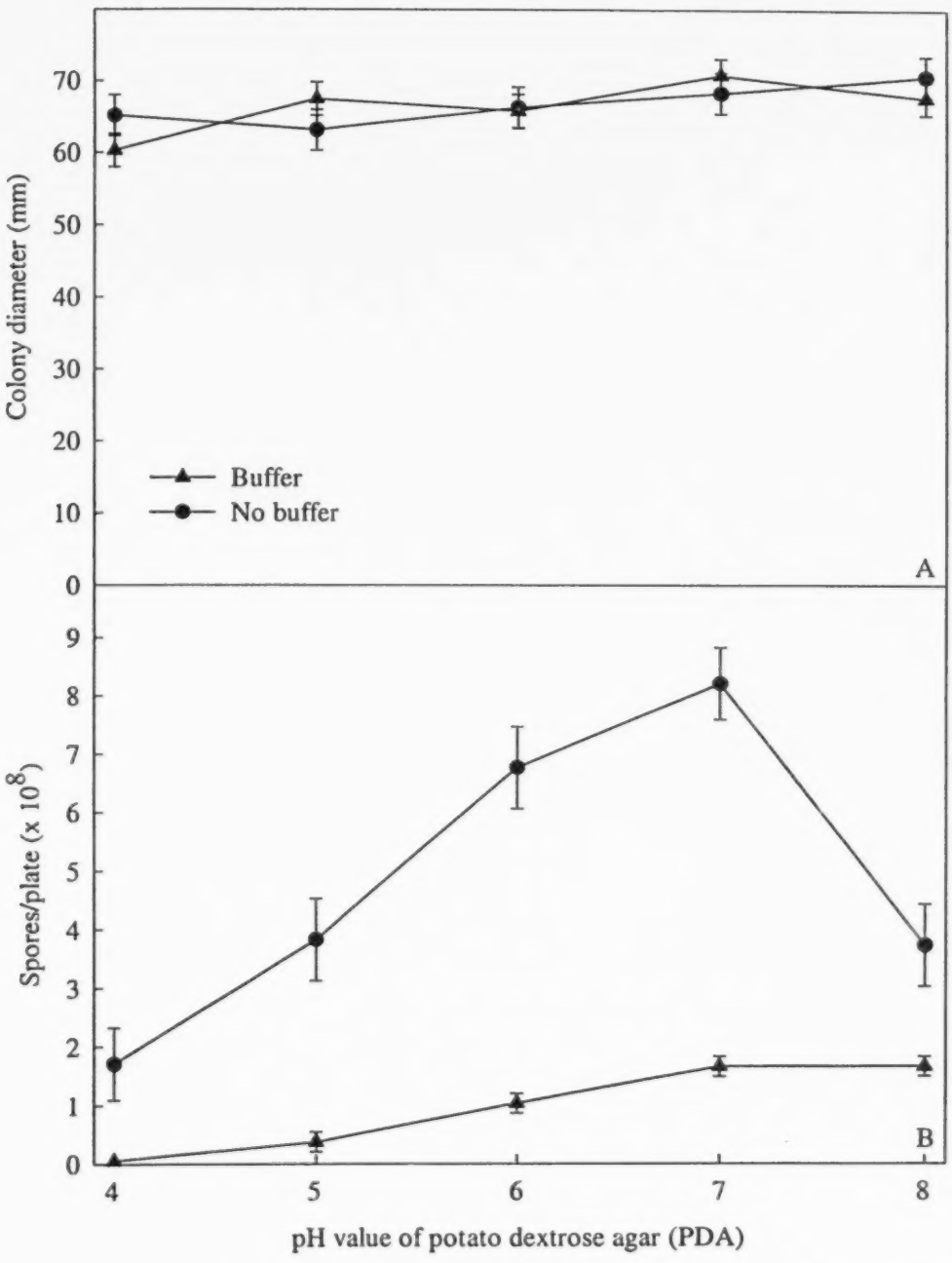

Figure 2.2. Effect of the pH of buffered and unbuffered potato dextrose agar media on the radial mycelial growth (A) and conidia production (B) of *Plectosporium tabacinum*. The number of conidia per plate was determined after 21 d incubation while mycelial growth was measured after 14 d incubation. Results of two trials with three replicates per trial were combined for each medium. Standard errors are indicated by vertical bars.

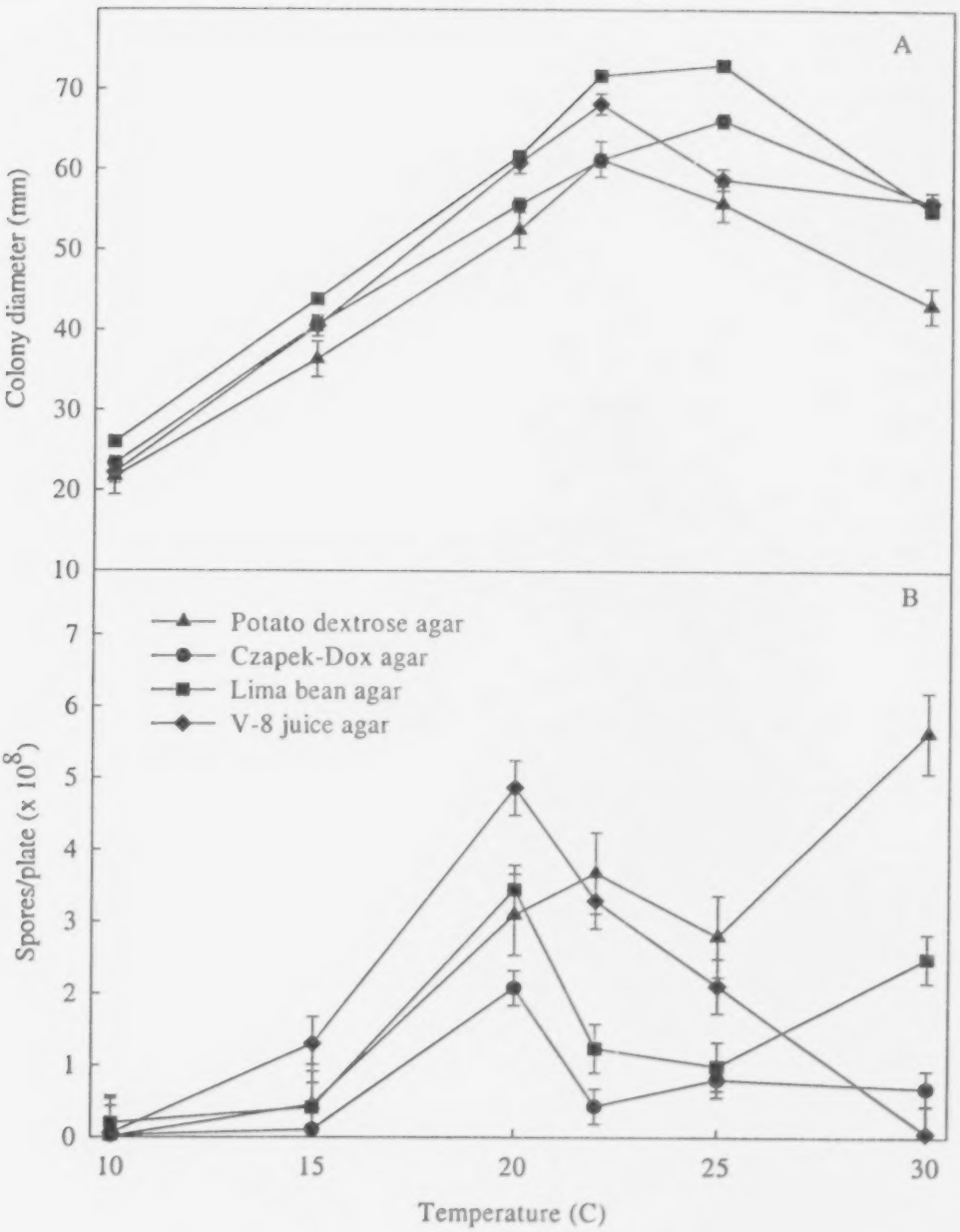

Figure 2.3. Effect of temperature on the radial mycelial growth (A) and conidia production (B) of *Plectosporium tabacinum* on potato dextrose agar (PDA), Czapek Dox agar (CDA), lima bean agar (LBA) and V-8 juice agar (VA). The number of conidia per plate was determined after 21 d incubation while mycelial growth was measured after 14 d incubation. Results of two trials with three replicates per trial were combined for each medium. Standard errors are indicated by vertical bars.

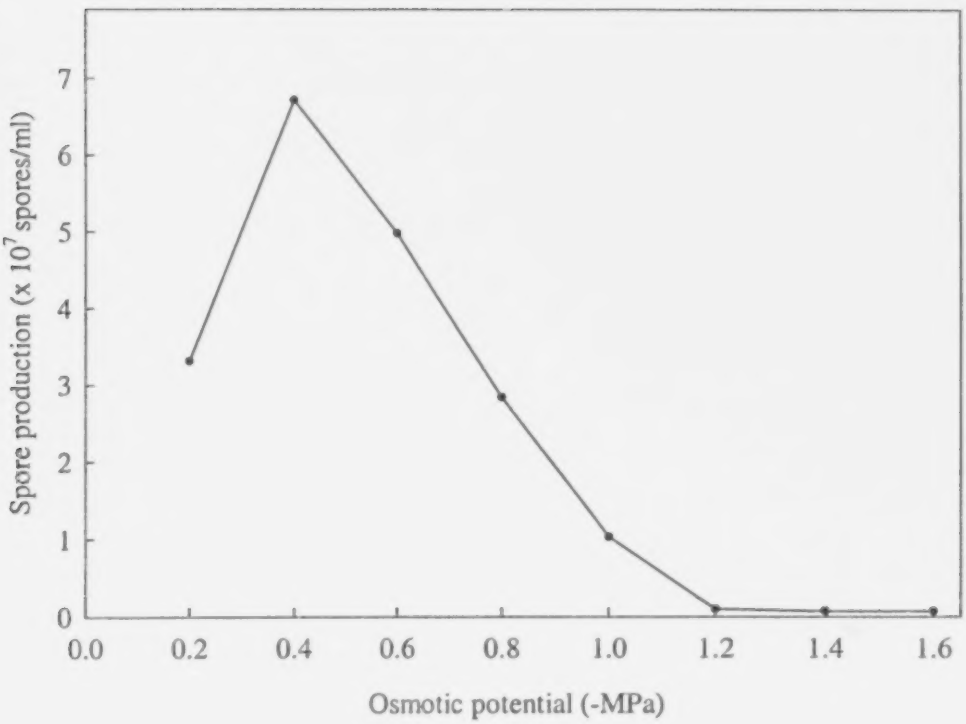

Figure 2.4. Effect of osmotic potential on spore production of *Plectosporium tabacinum*. The number of spores/ml was determined after 72 h incubation on an orbit shaker at 150 rpm.

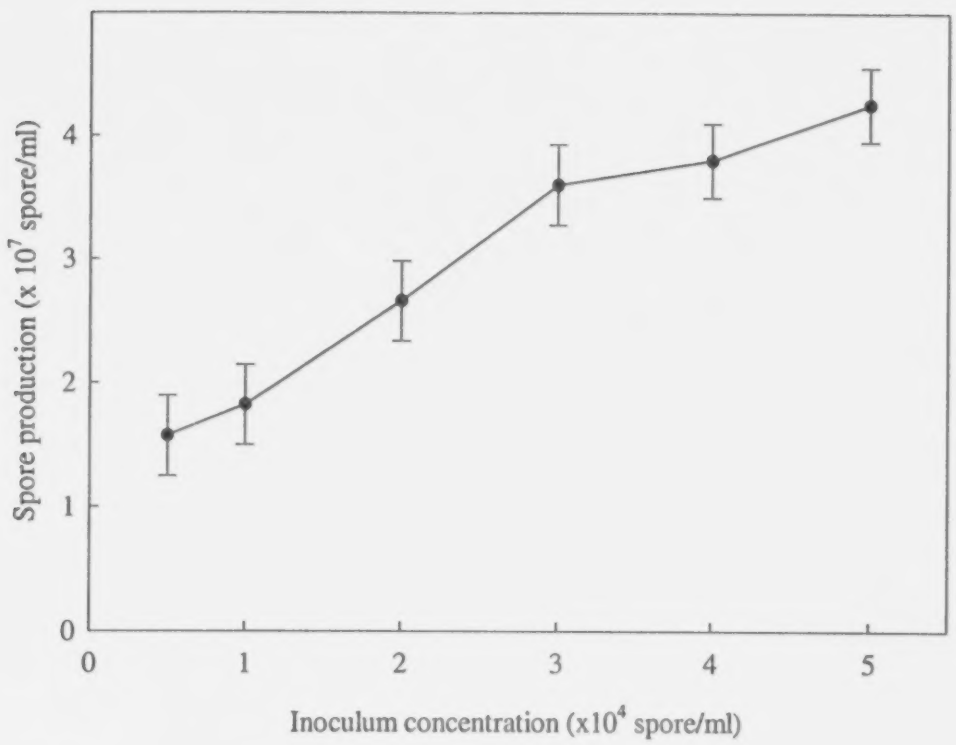

Figure 2.5. Effect of initial spore concentration on spore production of *Plectosporium tabacinum* in Richard's solution. The number of spores/plate was determined after 3 days incubation on an orbit shaker at 150 rpm.

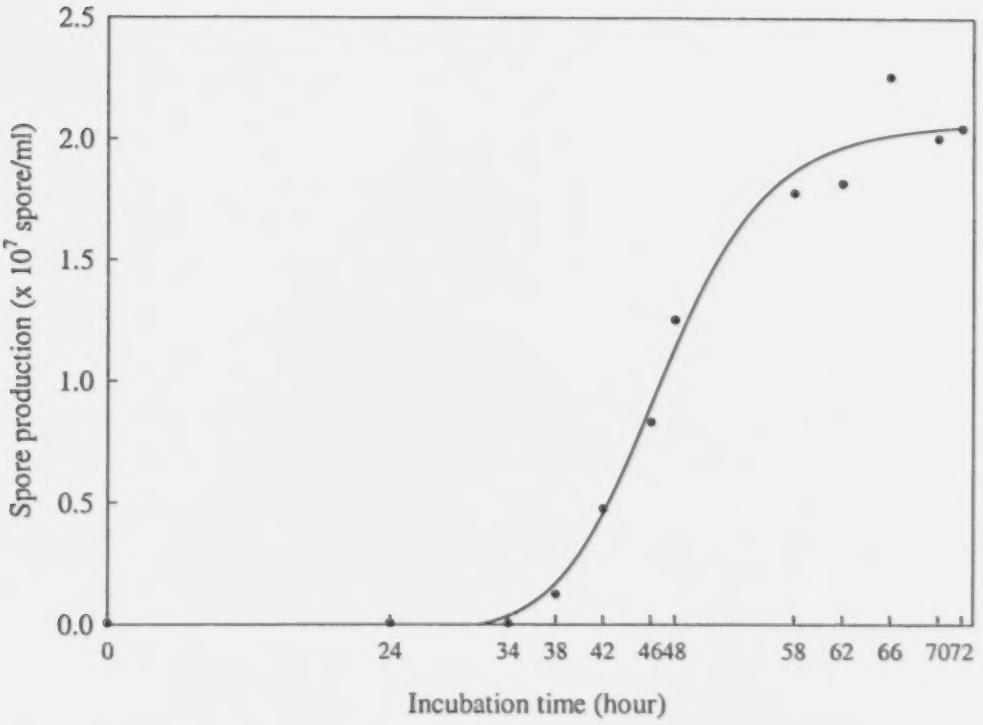

Figure 2.6. Spore production over incubation time of *Plectosporium tabacinum* in Richard's solution. The number of spores/plate was determined after 3 days incubation on an orbit shaker at 150 rpm.

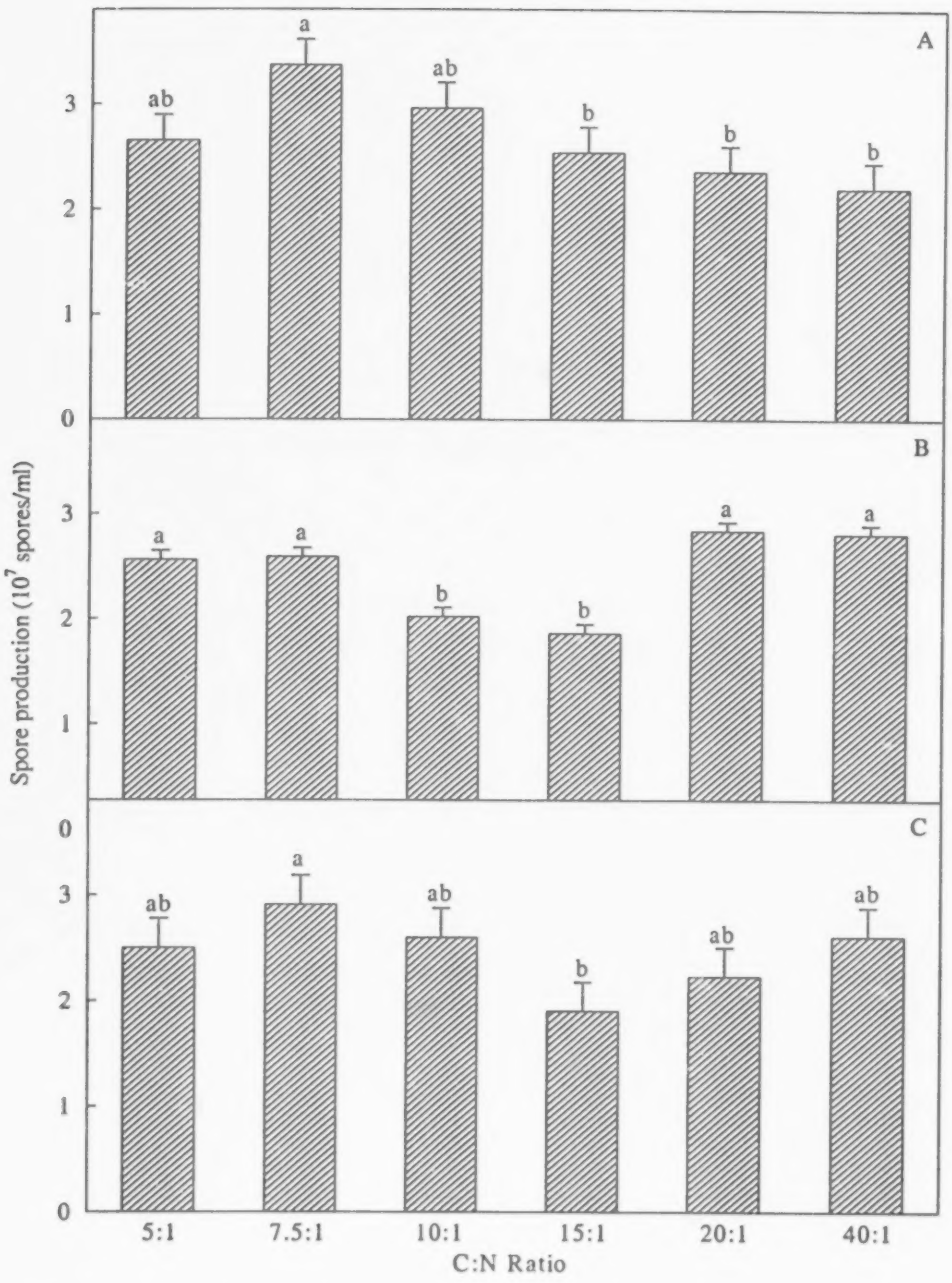

Figure 2.7. Effect of carbon-to-nitrogen ratio on spore production of *Plectosporium tabacinum* in a basal salt medium of Richard's solution. Media with three carbon (sucrose) and nitrogen (KNO_3) concentrations and six different ratios of carbon to nitrogen (C:N) was prepared. Media with sucrose concentrations of 8.4 (A), 21 (B), and 33.6 (C) g L^{-1} and C:N rations of 40:1, 20:1, 15:1, 10:1, 7.5:1, and 5:1 were prepared. Spore production in unmodified Richard's solution (50 g L^{-1} sucrose and a 5:1 C:N ratio) was included as a control. The number of spores/plate was determined after 3 days incubation on an orbit shaker at 150 rpm.

Chapter 3 Host Range of *Plectosporium tabacinum*

3.1. Abstract

A new strain of the fungus *Plectosporium tabacinum* was isolated from naturally infected false cleavers plants and evaluated as a bioherbicide for the control of both herbicide-resistant and herbicide-susceptible false cleavers in canola. *P. tabacinum* was non-pathogenic to both Argentine canola and Polish canola and to both herbicide tolerant and conventional cultivars. *P. tabacinum* killed false cleavers seedlings when applied at a concentration of 1 x 10^7 conidia ml^{-1} and provided with 16 h dew. The efficacy of *P. tabacinum* on herbicide-resistant false cleavers was identical to that on herbicide-susceptible false cleavers. Cleavers was also highly susceptible to *P. tabacinum*. Further host range tests on 34 plant species in 26 genera and 12 families demonstrated that *P. tabacinum* is sufficiently host specific for false cleavers control in western Canada. This fungus may provide a novel approach to managing herbicide-resistant false cleavers.

3.2. Introduction

False cleavers (*Galium spurium* L.) is a weed of important economic impact in western Canada, especially for producers of canola (*Brassica napus* L. and *B. rapa* L.) (Malik and Vanden Born, 1987a, 1987b, 1988). Weed surveys in the prairie provinces of Canada have demonstrated that false cleavers populations have increased during the past 10 years and its abundance ranking have increased more rapidly than any other cropland weed (Thomas et al., 1998a, 1998b, 1998c). Overall, the rankings of false cleavers for relative weed abundance rose by 36, 27, and 19 rankings in Alberta, Saskatchewan and Manitoba, respectively (Thomas et al., 1998a, 1998b, 1998c). Heavy infestations of false cleavers in canola cause severe yield losses, up to 18% with 100 false cleavers plants m^{-2} through crop/weed competition (Malik and Vanden Born, 1987b). Another significant impact of false cleavers in canola is the similarity in shape and size of the weed and crop seeds, making mechanical seed separation difficult (Malik and Vanden Born, 1988). False cleavers seed contamination in canola leads to downgrading of canola quality, has implications for the crushing industry, and contributes to the spread of weed infestations. Under the legislation in the Canada Seeds Act, no false cleavers seed is allowed in pedigreed canola seed and thus pedigreed seed producers of canola cannot tolerate land infested with this weed. False cleavers are not only difficult to control in canola but are an

increasing problem in other major crops of Western Canada including spring wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and pea (*Pisum sativum* L.).

Chemical herbicides used for false cleavers control include several acetolactate synthase (ALS) inhibitors along with an auxin-type herbicide combination. Unfortunately, multiple herbicide resistance has been detected in populations of false cleavers (Hall et al., 1998). This resistant false cleavers biotype shows cross-resistance to quinclorac and ALS inhibitors, including imazethapyr, one of the products for which herbicide-tolerant canola has been developed. With continuing herbicide use and herbicide-tolerant canola cultivation (approximately 20-40% or more of the canola acreage in Canada), herbicide resistance will become more common in false cleavers.

Successful weed control does not rely on a single weed management option, so alternative or new false cleavers control strategies are needed for both conventional and herbicide tolerant canola. The use of biological control agents for weed control may complement herbicide use by introducing novel modes of action to mitigate herbicide resistance development (Charudattan, 1991; Mortensen, 1998). Recently, biological control programs using pathogenic fungal pathogens as bioherbicides (Zhang and Bailey, 1999) or insects (a gall mite *Cecidophyes rouhollahi* Craemer) as classical biocontrol agents (Craemer et al., 1999) have been initiated. For bioherbicide approach, various fungal isolates were isolated from diseased leaves, stems, flowers and seeds of false cleavers. After screening these fungal pathogens for efficacy of false cleavers control and host specificity to major crops, a strain of the fungus, *Plectosporium tabacinum* (van Beyma) M. E. Palm, W. Gams et Nirenberg, was selected as a bioherbicide candidate for control of herbicide-resistant and herbicide-susceptible false cleavers in canola. This paper reports on the pathogenicity to false cleavers and canola, efficacy to false cleavers, and host specificity of *Plectosporium tabacinum*.

3.3. Materials and Methods

3.3.1. The pathogen.

P. tabacinum (isolate CL98-103, ARC) was isolated from diseased leaves of false cleavers growing in a crop of canola at Peace River, AB, Canada in 1998. Its identification was confirmed by W. Gams, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Conidia derived from single-conidium isolates recovered from infected host tissue were

placed in 2 ml of 15% glycerol solution in plastic cryovials and stored at –80 °C as stock cultures.

3.3.2. Inoculum production.

A cryovial of stock culture was warmed to room temperature in a water bath at 36°C, about 200 µl of suspension was aseptically placed on the surface of a PDA Petri plate and conidia were spread with a sterile glass rod. The Petri plate was incubated for 5 d at 22°C under 12 h cool white fluorescent light provided by two, 20W, 60 cm long tubes placed 30 cm above the Petri plates. Single-conidium colonies were then made from the actively growing cultures and incubated under the same conditions. Conidia from the single-conidium cultures were aseptically spread onto PDA and incubated under the conditions described above for 15 d to increase inoculum. Conidia were harvested by flooding the plates with 10 ml of distilled water and scraping the surface of the colonies with a glass slide. The resulting pooled suspensions were filtered through two layers of cheesecloth and conidial concentrations were determined with a hemacytometer.

3.3.3. Pathogenicity of *P. tabacinum* to false cleavers and canola.

For canola, two different species with or without novel traits were included in this test. Three herbicide-tolerant cultivars, Invigor 2153 (Liberty Link), Quest (Roundup Ready), and 45A71 (Pursuit Smart), and two conventional cultivars, Quantum and Impulse, were selected as representatives of Argentine canola, while Hysin 111 and Reward were selected as representatives of Polish canola. For false cleavers, both herbicide-resistant and herbicide-susceptible biotypes were included in this test. Herbicide-resistant false cleavers seeds were supplied by Dr. Linda Hall at Alberta Agriculture, Food, and Rural Development, Edmonton, AB, Canada, while herbicide-susceptible false cleavers seeds were obtained from Dr. Alec McClay at the Alberta Research Council, Vegreville, AB, Canada. Seven to 10 seeds of herbicide-resistant or herbicide-susceptible false cleavers and canola cultivars were sown in each 10 cm diameter peat pot containing steam-pasteurized soil mix (140 L soil, 107 L sand, 160 L Sunshine Growing Mix[1], 62 L Fibrous Blond Sphagnum Peat Moss[2], 113 L vermiculite[3], 230 g dolomite lime, 150 g Super Phosphate). Seeded pots were placed in a greenhouse with 24/20 ± 5°C day/night temperature, a 16 h photoperiod, an average light intensity of 300 $\mu Em^{-2}s^{-1}$, and an

average relative humidity of 45-50%. After germination, seedlings were thinned to 5 plants per pot. False cleavers seedlings at the one-whorl stage and canola seedlings at the one- or two-leaf stage were sprayed to run-off with 10^6 to 10^7 conidia per ml in 1% gelatin solution[4], using an H-set airbrush[5] at a pressure of 100 kPa. Control plants were sprayed with 1% gelatin only. About 30 min after spraying, pots were placed in a dark dew chamber[6] at 100% relative humidity at 22°C for 24 h. Subsequently, pots were returned to the greenhouse for the remainder of the experiment. The disease reactions of herbicide-resistant and herbicide-susceptible false cleavers and canola to *P. tabacinum* were evaluated seven days after inoculation. On the basis of lesion type and size, plant response was rated at four levels: 0 = lesions absent; 1= small, unexpanded lesions; 2= slightly to moderately expanded lesions; and 3= large lesions or dead plants. The experimental design was a randomized complete block with four replications (five plants per replication). This experiment was performed twice. The results from the two trials of the experiment were pooled because the variances of the trials were homogenous after the Bartlett's test (Gomez and Gomez, 1984).

3.3.4. Host range of *P. tabacinum*.

Using the modified centrifugal phylogenetic and varietal strategy (Wapshere, 1974), 35 plant species in 26 genera and 12 families were selected for the host range trial (Table 3.1). Test plants were grown from seed or propagated vegetatively depending on the species being tested and availability of material. For each species, two sets of test plants (each set containing 15 plants) were prepared. One set was inoculated with *P. tabacinum* and the other set served as uninoculated controls. For the inoculated treatment, seedlings of selected plant species at the 2- to 3-leaf stage were sprayed to run-off with 1×10^7 conidia per ml in 1% gelatin solution, using the inoculation method described above. For uninoculated control, seedlings of selected plant species at the 2- to 3-leaf stage were sprayed with 1% gelatin. About 30 min after spraying, pots were placed in the dark dew chamber with 100% relative humidity at 22°C for 24 h. Subsequently, pots were returned to the greenhouse for the remainder of the experiment. Greenhouse conditions were the same as described above. Disease severity was visually assessed daily until the 14^{th} day after inoculation by using the 0-11 rating scale by Horsfall and Barrett (1945). False cleavers seedlings at the 1- whorl stage were also inoculated with the same inoculum and

served as a control. The experiment was conducted as a factorial experiment with species tested and inoculation levels (0 and 1 x 10^7 conidia per ml) as factors. Each treatment was replicated three times and pots were placed randomly both in the dew chamber and greenhouse.

3.4. Results

3.4.1. Pathogenicity of *P. tabacinum* to false cleavers and canola.

P. tabacinum caused large, necrotic lesions on leaves and stems of both herbicide-resistant and herbicide-susceptible false cleavers seedlings. The disease rating was 3 on both herbicide-resistant and herbicide-susceptible false cleavers (Table 3.2). The disease reaction on false cleavers was characterized by a diffuse water-soaking reaction, followed by rapid collapse and necrosis of affected tissues (Figure 3.1). *P. tabacinum*, however, was not pathogenic to five cultivars of Argentine canola and two cultivars of Polish canola (Table 3.2).

3.4.2. Host range of *P. tabacinum*.

Host specificity of a biocontrol agent is another important factor to consider in a biological weed control program (Watson, 1985). Before using a biological control agent in the field, the safety of non-target economic and wild plants must be assured (Wapshere, 1974). The host range test including 35 plant species in 26 genera and 12 families demonstrated that this strain of *P. tabacinum* is not pathogenic to most plant species tested. Table 3 shows the plant species with disease ratings greater than one.

All seedlings of false cleavers serving as positive controls were killed when subjected to a 24 h dew period. Cleavers (*Galium aparine* L.), another weed species in the *Galium* weed complex in western Canada (Malik and Vanden Born, 1988), was also killed with 24 h dew. The findings in this study demonstrated that our strain of *P. tabacinum* could be used for the control of two weedy *Galium* species. Results also showed that other species in the genus *Galium* are immune to *P. tabacinum*. Hosts of *P. tabacinum* in the Rubiaceae family were restricted to the Section Kolgyda (false cleavers and cleavers) in the Genus *Galium*, the tribe Rubieae, and the subfamily Rubiodieae. The use of this strain of *P. tabacinum* should therefore be safe to non-weedy native *Galium* species and other plant species in this family.

3.5. Discussions

The results indicate that canola is not a host of *P. tabacinum*. This is consistent with reports by Farr et al. (1989) and Ginns (1986). Thus, it is reasonable to conclude that it is safe to use *P. tabacinum* for the control of false cleavers in canola.

P. tabacinum caused slight symptoms with limited expansion of lesions on sunflower (*Helianthus annuus* L.) with the 24 h dew period treatment. Lesion expansion covered less than 10% of inoculated leaves 2 weeks after inoculation and no stem lesions were observed. Lesions did not spread to emerging uninoculated leaves. Similarly, *P. tabacinum* caused small (<0.5 mm) brown flecks on inoculated leaves of flax (*Linum usitatissimum* L.), safflower (*Carthamus tinctorius* L.), melon (*Cucumis melo* L.), zucchini (*Cucubita pepo* L.), and tomato (*Lycopersicon esculentum* Mill.) with a 24 h dew period treatment. However, these small flecks did not expand during the 2-week observation period. Lesions covered less than 3% of infected leaves. It has been reported that *P. tabacinum* caused diseases of sunflower and basil (*Ocimum basilicum* L.) in Italy (Matta, 1978; Zazzerini & Tosi, 1987), melon and zucchini in the southeastern United States (Bost & Mullins, 1992; Saad & Black, 1981), tomato in Australia (Pascoe *et al.*, 1984), and tobacco (*Nicotiana tabacum* L.) in The Netherlands (Palm et al., 1995). In Canada, *P. tabacinum* has been isolated from several substrates at various locations and on different occasions in Quebec, Ontario, Saskatchewan, Alberta, and British Columbia (Seifert, 1996). For instance, *P. tabacinum* has been isolated from soil, roots of giant ragweed (*Ambrosia trifida* L.), ginseng (*Panax quinquefolius* L.), potato (*Solanum tuberosum* L.), and dandelion (*Taraxacum officinale* Weber *in* Wiggers), and seeds of ginseng. However, *P. tabacinum* has never been reported causing crop diseases in Canada (Ginns, 1986; Seifert, 1996). Whether this is because the western Canadian climate conditions are unsuitable for natural infection of this pathogen on these susceptible crops, or because these minor crops are planted in relatively small acreage, is unknown. The detection of natural infection of *P. tabacinum* on false cleavers but not on any crops in western Canada suggests that this fungus can be used as a bioherbicide to control false cleavers with little or no chance of infection on crops that were reported to be hosts of *P. tabacinum* in other countries. Nevertheless, a detailed risk analysis using

modeling procedures proposed by Jong et al. (1990) should be done to provide more insights into the risks involved in releasing *P. tabacinum* in a field environment.

This is the first report of *P. tabacinum* as a pathogen of *Galium* species in Canada. The same fungus is also being investigated as a potential bioherbicide for arrowhead (*Sagittaria trifolia* L.), a weed difficult to control in rice field in Korea (Chung et al., 1998) and hydrilla (*Hydrilla verticillata* (L.F.) Royle), an invasive aquatic weed in the southeastern United States (Smither-Kopperl, 1999). Our strain of *P. tabacinum* may be different from the other two isolates found on arrowhead and hydrilla because there are differences in growth characteristics and host specificity between these isolates (Chung et al., 1998; Smither-Kopperl, 1999). Similarly, eleven other isolates of this fungus have also been isolated from common chickweed (*Stellaria media* (L.) Vill.), turfgrass, and soils in our lab, but there are differences in morphology, pathogenicity, virulence, growth rate, and sporulation among the twelve isolates (data not shown). Palm et al. (1995) suggest that *P. tabacinum* be further divided into subgroups. Whether or not these isolates can be differentiated at either forma specialis or race level remains to be seen. DNA fingerprinting might provide an approach for further comparison.

In summary, the fungal isolate in this study may be a new strain of the fungus *P. tabacinum*. This fungal isolate has unique characteristics and has potential to control two weedy *Galium* species in western Canada. In addition, this fungus may provide a novel approach to manage herbicide-resistant false cleavers.

3.6. Literature Cited

1. Bost, S.C. and C.A. Mullins. 1992. A blight of cucurbits caused by *Microdochium tabacinum*. (Abstr.) Plant Dis. 76: 861.
2. Charudattan, R. 1991. The mycoherbicide approach with plant pathogens. Pages 24-57 *in* D.O. TeBeest, ed. Microbial Control of Weeds. New York: Chapman & Hall.
3. Chung, Y.R., S.J. Koo, H.T. Kim, and K.Y. Cho. 1998. Potential of an indigenous fungus, *Plectosporium tabacinum*, as a mycoherbicide for control of arrowhead (*Sagittaria trifolia*). Plant Dis. 82: 657-660.
4. Craemer, C., Sobhian, R., McClay, A.S. and J.W. Amrine. 1999. A new species of *Cecidophyes* (Acari: Eriophyidae) from *Galium aparine* (Rubiaceae) with notes on its

biology and potential as a biological control agent for *Galium spurium*. International J. Acarol. 25: 255-263.

5. Farr, D.F., G.F. Bills, G.P. Chamuris and A.Y. Rossman. 1989. Fungi of Plants and Plant Products in the United States. APS Press, St. Paul, Minn.
6. Ginns, J.H. 1986. Compendium of Plant Disease and Decay Fungi in Canada 1960-1980. Canadian Government Publishing Centre, Ottawa, Canada. Pp416.
7. Gomez, K.A. and A.A. Gomez. 1984. Statistical Procedures for Agricultural Research. 2nd ed. New York: J. Wiley, 680 p.
8. Hall, L.M, K.M. Stromme, G.P. Horsman, and M.D. Devine. 1998. Resistance to acetolactate synthase inhibitors and quinclorac in a biotype of false cleavers (*Galium spurium*). Weed Sci. 46:390-396.
9. Horsfall, J.G. and R.W. Barrett .1945. An improved grading system for measuring plant diseases. Phytopathol. 35:655.
10. Jong, M.D. de, P.C. Scheepens, and J.C. Zadocks. 1990. Risk analysis for biological control: a Dutch case study in biocontrol of *Prunus serotina* by the fungus *Chondrostereum purpureum*. Plant Dis. 74:189-194.
11. Malik, N. and W.H. Vanden Born. 1987a. Growth and development of false cleavers (*Galium spurium* L.). Weed Sci. 35:490-495.
12. Malik, N. and W.H. Vanden Born. 1987b. False cleavers (*Galium spurium* L.) competition and control in rapeseed. Can. J. Plant Sci. 67: 839-844.
13. Malik, N. and W.H. Vanden Born. 1988. The biology of Canadian weeds. 86. *Galium aparine* L. and *Galium spurium* L. Can. J. Plant Sci. 68: 481-499.
14. Matta, A. 1978. *Fusarium tabacinum* (Beyma) W. Gams patogeno in natura su basilico e pomodoro. Riv. Patol. Veg. 14:119-126.
15. Mortensen, K. 1998. Biological control of weeds using microorganisms. *In* G.J. Boland and L.D. Kuykendall, eds. Plant-Microbe Interactions and Biological Control. New York:Marcel Dekker, Inc. pp.223-248.
16. Palm, M.E., W. Gams, and H.I. Nirenberg. 1995. *Plectosporium*, a new genus for *Fusarium tabacinum*, the anamorph of *Plectosphaerella cucumerina*. Mycologia. 87: 397-406.
17. Pascoe, I.G., R.J. Nancorrow, and C.J. Copes. 1984. *Fusarium tabacinum* on tomato and other hosts in Australia. Trans. Br. Mycol. Soc. 83: 343-345.

18. Saad, A.T. and L.L. Black. 1981. A new fungus disease of cucurbits. (Abstr.) Phytopathol. 71: 902.

19. Seifert, K.A. 1996. *Plectosporium tabacinum*, fungi canadenses No. 333. Can. J. Plant Path. 18:309-311.

20. Smither-Kopperl, M.L., R. Charudattan, and R.D. Berger. 1999. *Plectosporium tabacinum*, a pathogen of the invasive aquatic weed *Hydrilla verticillata* in Florida. Plant Dis. 83: 24-28.

21. Thomas, A.G., B. Frick, and L.M. Hall. 1998a. Weed population shifts in Alberta. Agriculture and Agri-Food Canada, Saskatoon, pp. 1.

22. Thomas, A.G., B. Frick, R. van Acker, and D. Joosse. 1998b. Weed population shifts in Manitoba. Agriculture and Agri-Food Canada, Saskatoon, pp. 1.

23. Thomas, A.G., B. Frick, R.F. Wise, and L.T. Juras. 1998c. Weed population shifts in Saskatchewan. Agriculture and Agri-Food Canada, Saskatoon, pp. 1.

24. Wapshere, A.J. 1974. A strategy for evaluating the safety of organisms for biological weed control. Ann. App. Biol. 77:201-211.

25. Watson, A.K. 1985. Host specificity of plant pathogens in biological weed control. *In* E.S. Delfosse, eds. Proceedings of the 6th International Symposium on Biological Control of Weeds. Ottwa, Ont. Pp.577-586.

26. Watson, A.K. and L.A. Wymore. 1990. Identifying limiting factors in the biocontrol of weeds. *In* R.R. Baker and P.E. Dunn, eds. New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases. New York: Alan R. Liss, pp. 305-316.

27. Wiedeman, G.J. and D.O. TeBeest. 1990. Biology of host range testing for biocontrol of weeds. Weed Technol. 4:465-470.

28. Zazzerini, A. and L. Tosi. 1987. New sunflower disease caused by *Fusarium tabacinum*. Plant Dis. 71: 1043-1044.

29. Zhang, W. and K.L. Bailey. 1999. Biological control of cleavers (*Galium spurium* and *G. aparine*) with pathogenic fungi – exploration and discovery. *In* N.R. Spencer, eds. Proceedings of the X International Symposium on Biological control of Weeds. Bozeman, MT, pp.95-101.

Table 3.1. Test plant species used for host-specificity screening of *Plectosporium tabacinum* against false cleavers (*Galium spurium*) based on the modified centrifugal phylogenetic and varietal strategy

Rubiaceae	1. *Galium spurium* L. (False cleavers)	7. *G. trifidum* L. (Three petal bedstraw)
	2. *G. aparine* L. (Cleavers)	8. *Asperula arvensis* L. (Blue woodruff)
	3. *G. boreale* L. (Northern bedstraw)	9. *Sherardia arvensis* L. (Blue field madder)
	4. *G. triflorum* Michx. (Fragrant bedstraw)	10. *Houstonia longifolia* L. (Longleaf summer blue)
	5. *G. trifidum* L. (Three petal bedstraw)	11. *Cephalanthus occidentalis* L. (Button bush)
	6. *G. mollugo* L. (Common hedge bedstraw)	
Brassicaceae	12. *Brassica napus* L. (Argentine Canola)	
	cv. Invigor 2153 (Liberty Link)	cv. Quantum (Conventional)
	cv. Quest (Roundup Ready)	cv. Impulse (Conventional)
	cv. 45A71 (Pursuit Smart)	
	13. *Brassica rapa* L. (Polish Canola)	
	cv. Hysyn 111	*cv. Reward*
	14. *Brassica oleracea* L. var. botrytis L. (Cauliflower, cv. Snowball A)	
Poaceae	15. *Triticum aestivum* L. (Wheat, cv. Katepwa)	17. *Avena sativa* L. (Oats, cv. Unknown)

	16. *Hordeum vulgare* L. (Barley, cv. Bridge)	
Fabaceae	18. *Pisum sativum* L. (Pea, cv. Radley)	20. *Lens culinaris* Medic. (Lentil, cv. Laird)
	19. *Medicago sativa* L. (Alfalfa, cv. Algonquin)	
Linaceae	21. *Linum usitatissimum* L. (Flax, cv. Norlin)	
Asteraceae	22. *Helianthus annuus* L. (Sunflower cv. S6140)	24. *Taraxium officinale* Weber in Wiggers (Dandelion)
	23. *Carthamus tinctorius* L. (Safflower cv. unknown)	
Solanaceae	25. *Solanum tuberosum* L. (Potato cv. Normondy	26. *Lycopersicon esculentum* L. (Tomato cv. Bush Beef Steak)
	cv. Russet burbank)	27. *Nicotiana tabacum* L. (Tobacco cv. Turkish)
Balsaminaceae	28. *Impatiens balsami* L. (Balsam)	
Apiaceae	29. *Apium graveolens* L. (Celery cv. Utah Tall Green)	30. *Pastinaca sativa* (Parsnip cv. All American)
Violaceae	31. *Viola odorata* (English violet)	
Cucurbitaceae	32. *Cucumis melo* L. (Melon cv. Hales Best)	34. *Cucurbita pepo* L. (Pumpkin cv. Big Mac)
	33. *Cucurbita pepo* L. (Zucchini cv. Summer Squash)	
Asclepiadaceae	35. *Asclepias* sp. (Milkweed)	

Table 3.2. Pathogenicity of *Plectosporium tabacinum* on herbicide-resistant and herbicide-susceptible false cleavers and various cultivars of canola*

Plants	Host responses**
Galium spurium (False cleavers)	
Herbicide resistant type	3
Herbicide susceptible type	3
Brassica napus (Argentine canola)	
cv. Invigor 2153 (Liberty Link)	0
cv. Quest (Roundup Ready)	0
cv. 45A71(Pursuit Smart)	0
cv. Quantum (Conventional)	0
cv. Impulse (Conventional)	0
Brassica rapa (Polish canola)	
Hysin 111	0
Reward	0

* False cleavers seedlings at the 1-whorl stage and canola seedlings at the 1- to 2-leaf stage were inoculated with 10^6 to 10^7 spores/ml of *P. tabacinum*, placed in a dew chamber at 22 °C for 24 h and subsequently maintained in a greenhouse.

** Host responses to *P. tabacinum* were rated seven days after inoculation with a 0 to 3 grading system, where 0 = lesions absent and 3 = large expanded lesions.

Table 3.3. Disease severity results of host specificity screening for *Plectosporium tabacinum*

Test plant species*	Disease grade**
False cleavers (*G. spurium*)	11
Cleavers (*G. aparine*)	11
Sunflower (*Helianthus annuus*)	3
Flax (*Linum usitatissimum*)	1
Safflower (*Carthamus tinctorius*)	1
Melon (*Cucumis melo*)	1
Zucchini (*Cucurbita pepo*)	1
Tomato (*Lycopersicon esculentum*)	1

* All other species remained uninfected, severity ratings were 0.

** Seedlings of plants at the 2- to 3-leaf stage were inoculated with 10^6 to 10^7 spores/ml to run-off, placed in a dew chamber at 22 °C for 24 h and subsequently maintained in a greenhouse. Disease severity was visually assessed daily until the 14th day after inoculation by using the 0-11 rating scale by Horsfall and Barrett (1945).

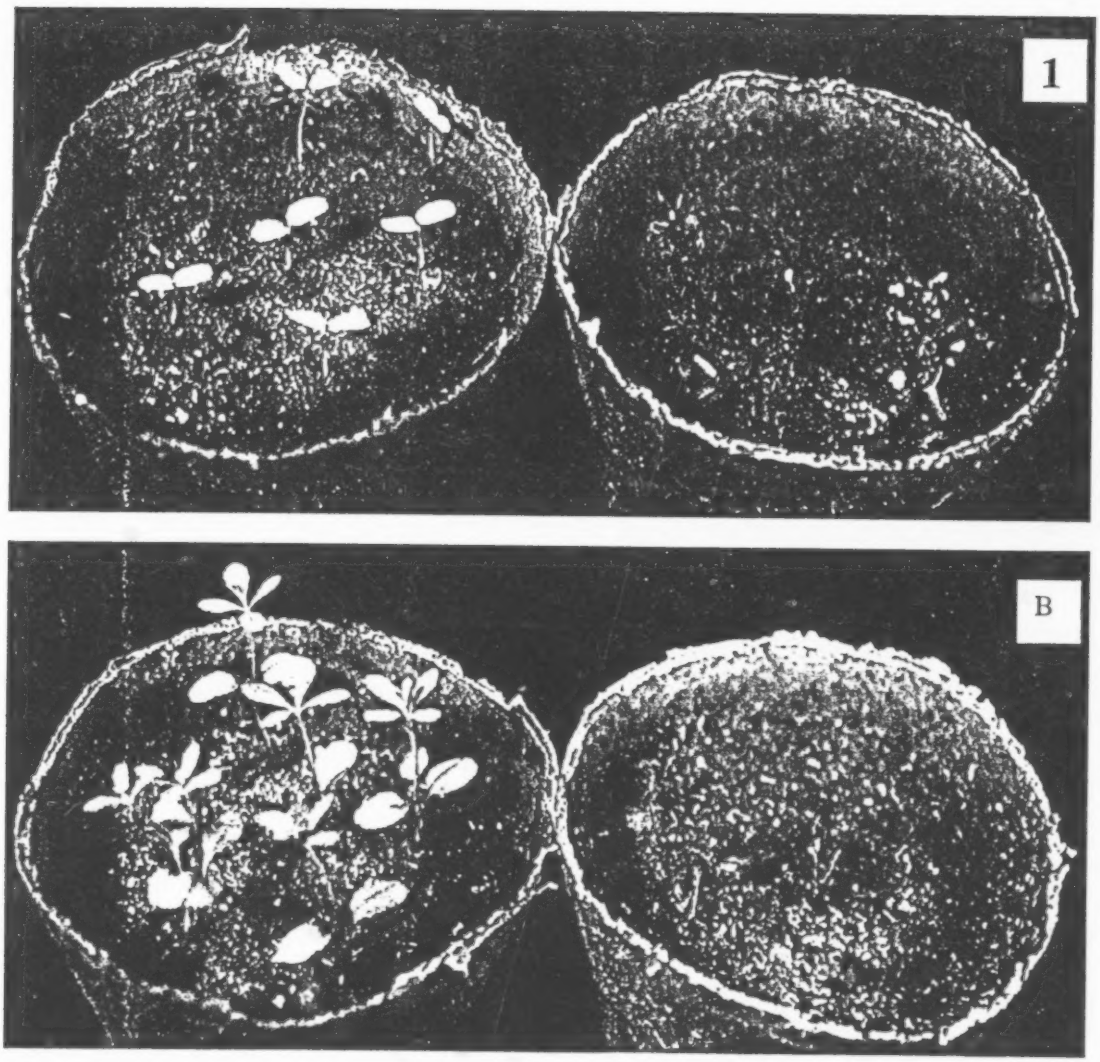

Figure 3.1. The disease reaction of false cleavers caused by the fungal pathogen *Plectosporium tabacinum*. (A) False cleavers at cotyledon stage and (B) False cleavers at 1-whorl stage. Left – Control and Right – Treated.

Chapter 4 Determination of Optimum Conditions and Limiting Factors for Maximum Effectiveness of *Plectosporium tabacinum* for Control of Cleavers (*Galium spurium*)

4.1. Abstract

The fungus *Plectosporium tabacinum* has been evaluated as a bioherbicide for control of false cleavers (*Galium spurium*), but the limiting factors and optimal conditions for successful control using this pathogen are not known. False cleavers mortality and dry weight reductions caused by *P. tabacinum* were assessed under single or combined factors, including pathogen inoculum concentration, plant growth stage, dew period (duration, frequency, and timing), and dew period temperature. The minimum inoculum concentration required to kill false cleavers seedlings was 1 x 10^7 conidia ml^{-1} at an application rate of 150 ml m^{-2}. False cleavers seedlings in the cotyledon or 1-whorl growth stage were the most susceptible. Increasing inoculum concentration increased weed control efficacy on older false cleavers seedlings. When an adequate dew period was provided, 100% mortality and dry weight reduction occurred. The minimum dew period to achieve 100% mortality was 16 h. Delaying the initiation of the dew period by 24 h significantly reduced disease development. Short, repetitive dew periods only improved false cleavers control to a limited extend and the length of initial dew is a critical factor influencing false cleavers mortality due to *P. tabacinum*. The optimal dew period temperature for disease development was above 15 °C.

4.2. Introduction

False cleavers (*Galium spurium* L.) is an important weed of canola (*Brassica napus* L. and *B. rapa* L.) and other crops in western Canada (Malik & Vanden Born, 1988). There has been a dramatic increase in the abundance of false cleavers across the prairie provinces of Canada during the past 10 years (Thomas *et al.*, 1998a, 1998b, 1998c) and an increase in the false cleavers biotypes that are resistant to the commonly used Group-2 and Group-4 herbicides (Hall *et al.*, 1998). Biological control of false cleavers using pathogenic fungi in an inundative approach may provide an alternate control option to herbicides (Zhang & Bailey, 2000). The native fungal pathogen, *Plectosporium tabacinum* (van Beyma) M. E. Palm, W. Gams et Nirenberg, kills false cleavers but is non-pathogenic to canola, six other major crops growing in western Canada, and 27 other

plant species (Zhang et al., 2001), demonstrating that this pathogen could be a potential bioherbicide against false cleavers (Zhang & Sulz, 2001).

In nature, infection and disease development of bioherbicides or other fungal pathogens is frequently suppressed (Watson & Wymore, 1990; TeBeest, 1991), so it is necessary to determine the optimal conditions that can be taken advantage of, and the limiting factors that must be overcome, to get effective control of false cleavers by *P. tabacinum* in the field. Pathogen inoculum concentration, weed growth stage, dew period duration, and temperature have been recognized as important factors affecting the efficacy of bioherbicides (TeBeest, 1991; Yang & TeBeest, 1993). The objective of this study was to determine the optimum conditions and limiting factors affecting disease development and control of false cleavers with *P. tabacinum*. Specific effects evaluated were 1) fungal inoculum concentration; 2) weed growth stage; 3) dew period duration, frequency, and timing; and 4) temperature during the dew period.

4.3. Materials and Methods

4.3.1. Inoculum production.

Stock culture of *P. tabacinum* were stored at -80 °C in 2 ml of 15% glycerol solution. A vial of stock culture was brought to room temperature and then 200 µl of the fungal suspension was spread on the surface of a 10-cm-diameter PDA Petri plate. The Petri plate was incubated for 5-8 days at 22°C under 12 h cool white fluorescent light provided by two, 20W, 60 cm long tubes placed 30 cm above the Petri plates.

Three agar plugs (5 mm diameter) or 1 ml of a conidial suspension (1×10^7 conidia/ml) were used to inoculate 200 ml Richard's solution (Tuite, 1969) in a 500 ml Erlenmeyer flask. Inoculated flasks were incubated under ambient laboratory conditions (24 ± 3°C) on an orbital shaker at about 150 rpm. After 3 d incubation, conidia were harvested by chopping the contents of each flask using an electric hand blender (Braun Multipractic MR 20, Lunnfield, MA, USA) and centrifuging 10 min to form a spore pellet (Sorvall RC-5B refrigerated superspeed centrifuge). The supernatant was decanted off and the conidial pellet was resuspended in an appropriate amount of 1% gelatin (Difco Laboratories, MI, USA) to achieve the desired inoculum concentration as determined with the aid of a haemocytometer. Unless otherwise stated, 1% gelatin

solution containing 0.5-1% macerated PDA was used as a wetting agent for all experiments.

4.3.2. Plant production.

Seedlings of false cleavers were grown by placing 7-10 seeds in each 10 cm diameter peat pot containing steam-pasteurized soil mix. Soil mix consisted of 140 L soil, 107 L sand, 160 L Sunshine Growing Mix (SunGro Horticulture, Bellevue, Washington), 62 L Fibrous Blond Shagnum Peat Moss (Premier Pro Moss, Rivere-du-Loup, Quebec), 113 L vermiculite (Therm-O-Rock, Chandler, Arizona), 230 g dolomite lime, 150 g Super Phosphate (0-45-0) per batch of soil. After germination, seedlings were thinned to 5 plants per pot. Greenhouse conditions throughout all experiments were 24/20 ± 5°C day/night temperature with a 16 h photoperiod.

4.3.3. Inoculation procedure.

False cleavers seedlings at the 1-whorl stage were inoculated with a conidial suspension at the concentration of $1x10^7$ spores/ml for all experiments (unless otherwise indicated) using an H-set airbrush (Paasche Airbrush Company, Harwood Heights, IL) at a pressure of 100 kPa. Each replicate pot received about 10 ml of inoculum. About 30 min after spraying, pots were placed in a dark dew chamber with 100% relative humidity at 22°C for 24 h or as indicated in the experiment. Pots were then returned to the greenhouse for the remainder of the experiment.

4.3.4. Assessment of disease development.

Mortality of plants and dry weight of aboveground biomass per pot were assessed seven days after inoculation. Completely collapsed seedlings were considered dead. Dry weight was obtained by cutting the aerial parts off at the soil level, drying in a paper bag for 48 h at 70°C, and weighing. The dry weight data were expressed as percent reduction in biomass compared with biomass of uninoculated controls.

4.3.5. Effect of inoculum concentration and plant growth stage.

Seedlings at the cotyledon, and 1-, 2-, 3-, 4-, or 5-whorl growth stage of false cleavers were inoculated with a conidial suspension at concentrations of 1×10^8, 1×10^7, 1×10^6,

1×10^5 or 0 spores/ml. After inoculation, pots with inoculated seedlings were placed in a dark dew chamber for 24 h and then returned to the greenhouse.

4.3.6. Effect of dew period duration.

After inoculation, pots with inoculated seedlings were placed in a dew chamber for 0, 4, 8, 12, 16, 20, or 24 h. Except for the 24-h treatment, all pots were covered with cardboard for the remainder of the 24 h to simulate the dark period in the dew chamber. After the dew period treatment, pots were returned to the greenhouse. Uninoculated pots receiving 0 and 24 h dew were included as controls.

4.3.7. Interaction of inoculum concentration and dew period duration.

False cleavers seedlings at the 1-whorl stage were inoculated with conidial suspension at a concentration of $5x10^6$, $1x10^7$, $5x10^7$, or $1x10^8$ conidia/ml, and were placed in the dew chamber for 9, 12, 16, or 20 h and then returned to the greenhouse.

4.3.8. Interaction of inoculum concentration and dew period temperature.

False cleavers seedlings at 1- whorl stage were inoculated with conidial suspension at a concentration of $1x10^7$, $1x10^6$, or $1x10^5$ conidia/ml. After inoculation, pots with inoculated seedlings were placed in the dew chamber at 100% humidity and temperatures of 10, 15, 20, or 30°C for 24 h and then returned to the greenhouse.

4.3.9. Effect of repetitive dew.

After inoculation, pots with inoculated seedlings were provided one of the following dark dew periods: (1) 0-h dew, (2) 8-h dew/16-h dry period on 1, 2, 3 or 4 consecutive days, (3) 12-h dew/12-h dry period on 1 or 2 consecutive days, (4) 16-h dew/8-h dry on 1 or 2 consecutive days; or (5) a single 24-h dew period. All pots were kept in the dark for the remainder of the 24 h to simulate the dark period in the dew chamber by covering pots with cardboard. After treatment, pots were returned to the greenhouse.

4.3.10. Effect of delaying initial dew period.

Pots with inoculated seedlings were placed in the greenhouse for 0, 1, 2, 3, or 4 days before being placed in a dark dew chamber for 4, 8, 12, 16, 20, or 24 h. The experiment had a 5 x 6 factorial design. Except for the 24-h treatment, pots removed from the dark

dew chamber were covered with cardboard for the remainder of the longest dew period of the day to simulate the dark period in the dew chamber. After treatment, pots were returned to the greenhouse.

4.3.11. Data analysis.

All experiments were conducted twice in a randomized complete block design with three replications of each treatment. All percentage data were arc sine-transformed before analysis (Gomez and Gomez, 1984). The results from the two trials of each experiment were not pooled because the variances of trials were not homogeneous after the Bartlett's test (Gomez and Gomez, 1984). However, a similar trend was observed between the trials. Thus, the results from one trial are presented. Mean values of five plants for each treatment were used for statistical analyses, and treatment means were separated using Duncan's multi-range test (DMRT) at the 5% level of significance.

4.4. Results

4.4.1. Effect of inoculum concentration and plant growth stage.

One hundred percent mortality of false cleavers at the cotyledon- to 1-whorl leaf stage and with an inoculum concentration of 10^7 conidia/ml or greater was observed (Figure 4.1). Increasing inoculum concentration increased mortality and percent dry weight reduction at all the plant growth stages tested. Younger plants at cotyledon and 1-whorl growth stages were more susceptible to *P. tabacinum* than those at the 2-5 whorl stage. The 2-whorl leaf stage was a critical point deterring plant kill. No false cleavers plants older than 1-whorl were killed, although dry weight of older false cleavers plants was significantly reduced.

4.4.2. Effect of dew period duration.

Both mortality and dry weight reduction of false cleavers increased concurrently as dew period increased (Figure 4.2). More than 16 h dew resulted in 90-100% mortality of false cleavers. Although no plant death was observed for the 0-, 4-, and 8-h dew period treatment, *P. tabacinum* caused more than 30% dry weight reduction of false cleavers.

4.4.3. Interaction of inoculum concentration and dew period duration.

P. tabacinum caused significant plant mortality and percent dry weight reduction of false cleavers seedlings with certain inoculum concentration and dew period duration combinations (Figure 4.3). A higher inoculum concentration and a longer dew period duration resulted in more disease on false cleavers. *P. tabacinum* caused 80% mortality with 5 x 10^6 conidia/ml and 20 h dew, whereas at 1 x 10^7 conidia/ml or greater concentrations with the same dew period killed all false cleavers seedlings. Reducing the dew period to 16 h resulted in 20% mortality at 5 x 10^6 conidia/ml, but more than 90% mortality was still observed with *P. tabacinum* at 1 x 10^7 conidia/ml or greater. With a 12 h dew, mortality of false cleavers was less than 60% at the highest conidium concentration, but the dry weight was reduced more than 90% when the inoculum concentration was equal to or greater than 5 x 10^7 conidia/ml. No plants were killed with an 8 h dew at any inoculum concentration tested and the largest dry weight reduction was 60%.

4.4.4. Interaction of dew period temperature and inoculum concentration.

Dew period temperature at or above 15 °C caused 100% mortality and dry weight reduction of false cleavers seedlings. Higher inoculum concentrations caused greater disease on false cleavers seedlings at all temperatures tested.

4.4.5. Effect of repetitive dew periods.

When provided with a continuous dew of 16-h or more, *P. tabacinum* caused 90-100% mortality of *G. spurium* (Table 4.1). Repetitive 8-h dew periods on consecutive days reduced the dry weight, but did not kill any *G. spurium* plants. However, two 12-h dew periods on consecutive days increased false cleavers mortality, causing 83% mortality compared with 53% mortality with a single 12-h dew period.

4.4.6. Effect of delaying initial dew period.

The highest mortality and the largest reduction in dry weight of *G. spurium* were obtained following the shortest delay in providing a dew period (Figure 4.4). When the initial dew period was delayed by 1 day or longer after inoculation, *G. spurium* mortality and percent dry weight reduction decreased sharply. Longer dew periods helped to increase false cleavers mortality and reduce dry weight.

4.5. Discussion

Low initial inoculum levels often contribute to the failure of epidemic disease development and persistence of the pathogens in weed populations (Holcomb, 1982). In this study, increasing the inoculum concentration of *P. tabacinum* increased efficacy on false cleavers seedlings, showing that this bioherbicide can cause an epidemic through manipulation of initial inoculum dose.

In western Canada, spring-seeded canola is normally sown between May 9 and 23. The emergence date, thus, is usually between May 18 and May 29. False cleavers usually begin to emerge at the end of May with peak emergence around mid-June. The average temperature and precipitation in May and June are about 10 - 16 °C, 30 – 100 mm, respectively (Alberta Environment, 1987). False cleavers seedlings are usually at the cotyledon or 1-whorl stage at this time and chemical herbicides are usually applied (Malik and Vanden Born, 1988). The findings in this study demonstrated that 1-whorl stage or younger false cleavers seedlings are the most susceptible to *P. tabacinum*. Therefore, the application window for *P. tabacinum* to control false cleavers is similar to that of the conventional postemergence chemical herbicides. Since increasing inoculum dose increased weed control on older false cleavers seedlings, it is likely that increasing *P. tabacinum* inoculum density may increase the application window for effective control of false cleavers seedlings. The optimum dew period temperature for *P. tabacinum* to control false cleavers was above 15 °C. Temperature may be a limiting factor for this fungus as bioherbicide used in Canada. One way to minimize the impact of this limitation is to apply this fungal pathogen in the daytime instead of late afternoon as suggested for most potential bioherbicides.

The minimum dew period to achieve 100% mortality of false cleavers seedlings was 16 h. This dew period requirement is similar to those reported for other bioherbicides including BioMal® (*Colletotrichum gloeosporioides* f.sp. *malvae*) (Makowski, 1993). TeBeest (1991) suggested that dew period requirements of 16 h or longer could limit the practical use of fungi as biological weed control agents. Therefore, as in other bioherbicide pathosystems, dew period may be a limiting factor for the control of false cleavers. Formulations with surfactants or humectants may overcome this limitation. For example, preliminary study demonstrated that addition of 0.1% Silwet L-77 decreased the minimum dew period for 100% mortality of *G. spurium* to 12 h. 8-h dew also caused

more than 70% mortality and over 80% dry weight reduction. Further study is warranted to select better adjuvants that overcome the dew requirement.

Repetitive dew periods have been reported to shorten the optimal dew period required for efficient control of *Senna obtusifolia* by *Alternaria cassiae* and *Echinochloa* species by *Exserohilum monoceras* (Walker and Boyette, 1986; Zhang and Watson, 1997). Similar results were observed in our study. Shorter repetitive dew periods, more closely simulating field conditions than a single long dew period, enhanced the control of false cleavers seedlings. However, these results need to be further confirmed under field conditions. Other studies on control of *Echinochloa* species using *E. monoceras* and sicklepod using *A. cassiae* showed that a delay of 1 or 2 days in the occurrence of dew was tolerated without adversely affecting the efficacy of the pathogen (Walker and Boyette, 1986; Zhang and Watson, 1997). However, it appears that an immediate initiation of the dew after inoculation is better for *P. tabacinum* to control false cleavers seedlings.

In summary, the minimum inoculum concentration required to kill false cleavers seedlings was 1×10^7 conidia/ml. The 1-whorl growth stage or younger false cleavers seedlings are the most susceptible to *P. tabacinum*. The minimum dew period to achieve 100% mortality is 16 h without any formulation. The optimal dew period temperature for disease development was above 15 °C. Dew period duration and temperature are two limiting factors to be overcome in the further development of *P. tabacinum* as a bioherbicide for control of false cleavers. Formulation and application time are considered as critical for the use of *P. tabacinum*.

4.6. Literature Cited

1. Alberta Environment. 1987. Climate of Alberta With Data for Yukon and Northwest Territories. Report for 1987. pp91.
2. Gomez, K. A. and Gomez, A.A. 1984. Statistical Procedures for Agricultural Research. 2nd Edition. John Wiley & Sons, Inc., New York.
3. Hall, L.M., Stromme, K.M. Horseman, G.P., and Devine, M.D. 1998. Resistance to acetolactate synthase inhibitors and quinclorac in a biotype of false cleavers (*Galium spurium*). Weed Sci. 46: 390-396.

4. Holcomb, G.E. 1982. Constraints on disease development. Pages 61-71 *in* Charudattan, R. and Walker, H.L. (eds) Biological Control of Weeds with Plant Pathogens. Wiley, New York.

5. Makowski, R.M.D. 1993. Effect of inoculum concentration, temperature, dew period, and plant growth stage on disease of round-leaved mallow and velvetleaf by *Colletotrichum gloeosporioides* f.sp. *malvae*. Phytopathol. 83: 1229-1234.

6. Malik, N. and W.H. Vanden Born. 1988. The biology of Canadian weeds. 86. *Galium aparine* L. and *Galium spurium* L. Can. J. Plant Sci. 68: 481-499.

7. TeBeest, D.O. 1991. Ecology and epidemiology of fungal plant pathogens studied as biological control agents of weeds. Pages 97-114 *in* TeBeest, D.O. (ed) Microbial Control of Weeds. Chapman & Hall, New York.

8. Thomas, A.G., Frick, B.L., Juras, L.T., Hall, L., van Acker, R. and Joose, D. 1998. Changes in weed distributions indicated by quantitative surveys in the prairie provinces of Canada over 10 years. Proceedings of the 38th Annual WSSA Conference, Chicogo, IL.

9. Tuite, J. 1969. Plant Pathology Methods: Fungi and Bacteria. Burgess Publishing Co., Minneapolis, Minnesota, USA.

10. Walker, H.L. and Boyette, C.D. 1986. Influence of sequential dew periods on biocontrol of sicklepod (*Cassia obtusifolia*) by *Alternaria cassiae*. Plant Dis. 70:962-963.

11. Watson, A.K. and Wymore, L.A. 1990. Identifying limiting factors in the biocontrol of weeds. Pages 305-316 *in* Baha, R.R. and Dunn, P.E. (eds) New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases. Alan R. Liss, Inc. New York.

12. Yang, X.B. and TeBeest, D.O. 1993. Epidemiological mechanisms of mycoherbicide effectiveness. Phytopathol. 83:891-893.

13. Zhang, W. and Watson, A.K. 1997a. Efficacy of *Exserohilum monoceras* for the control of *Echinochloa* species in rice (*Oryza sativa* L.). Weed Sci. 45:144-150.

14. Zhang, W. and Watson, A.K. 1997b. Effect of dew period and temperature on the ability of *Exserohilum monoceras* to cause seedlings mortality of *Echinochloa* species. Plant Dis. 81:629-634.

Table 4.1. Effect of repetitive dew periods on mortality and reduction in dry weight of *Galium spurium* inoculated with *Plectosporium tabacinum*[*]

Dew period (h/day)	Mortality (%)	Dry weight reduction (%)
0	0 c[**]	10.0 c
8	0 c	15.6 c
8;8	0 c	28.0 bc
8;8;8	0 c	40.4 bc
8;8;8;8	0 c	45.0 b
12	53 b	77.4 a
12;12	83a	90.1 a
16	90 a	92.4 a
24	100 a	100.0 a

[*] Seedlings of false cleavers at the 1-whorl growth stage were inoculated with a conidial suspension at a concentration of 1 x 10^7 conidia/ml containing 1% gelatin (Difco Laboratories, Detroit, MI, USA) and 0.5-1% macerated PDA. Data from two trials were not pooled because variances were not homogenous. Data represent means of four replicates.

[**] Values having the same letter are not significantly different according to DMRT at the 5% significance level.

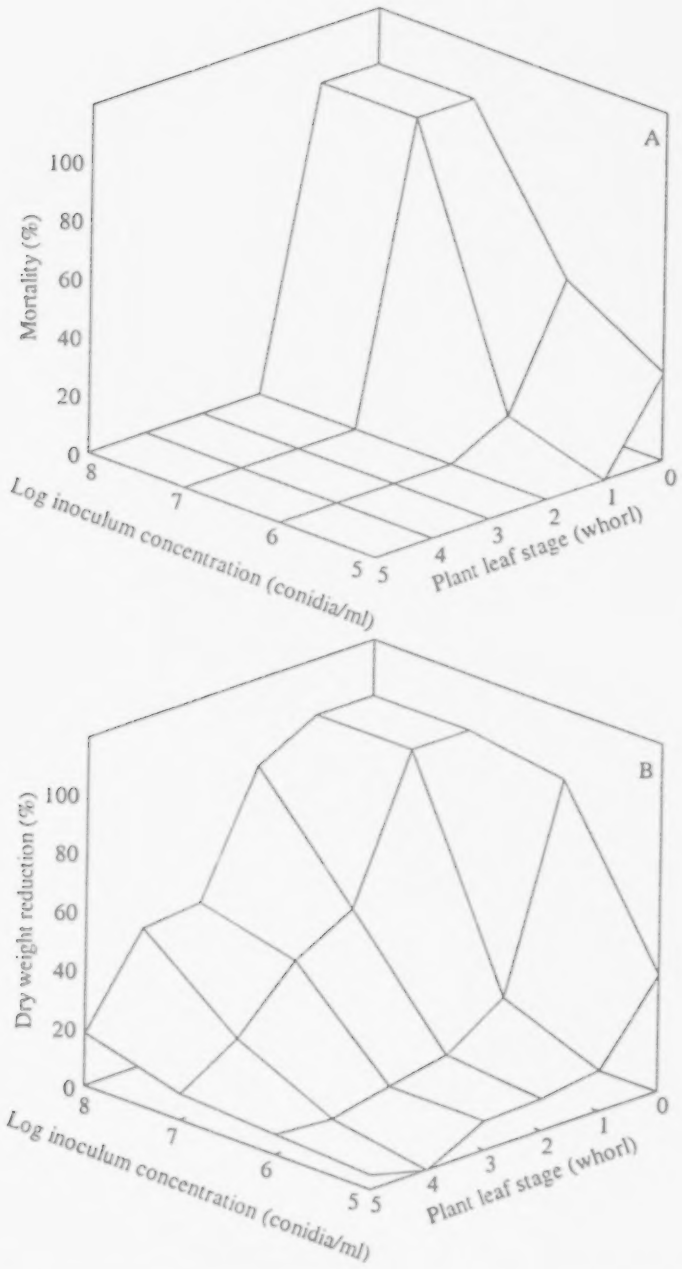

Figure 4.1. Effect of *Plectosporium tabacinum* inoculum concentration and plant growth stage on the control of false cleavers, expressed as percent mortality (A) and reduction in dry weight (B) seven days after inoculation. Seedlings of false cleavers at the cotyledon, and 1-, 2-, 3-, 4- or 5-whorl growth stage of false cleavers were inoculated with a conidial suspension at concentrations of 1×10^8, 1×10^7, 1×10^6, 1×10^5 or 0 spores/ml in 1% gelatin solution. After inoculation, pots with inoculated seedlings were placed in a dark dew chamber for 24 h. Data from two trials were not pooled because variances were not homogenous. Data represent the mean of three replicates.

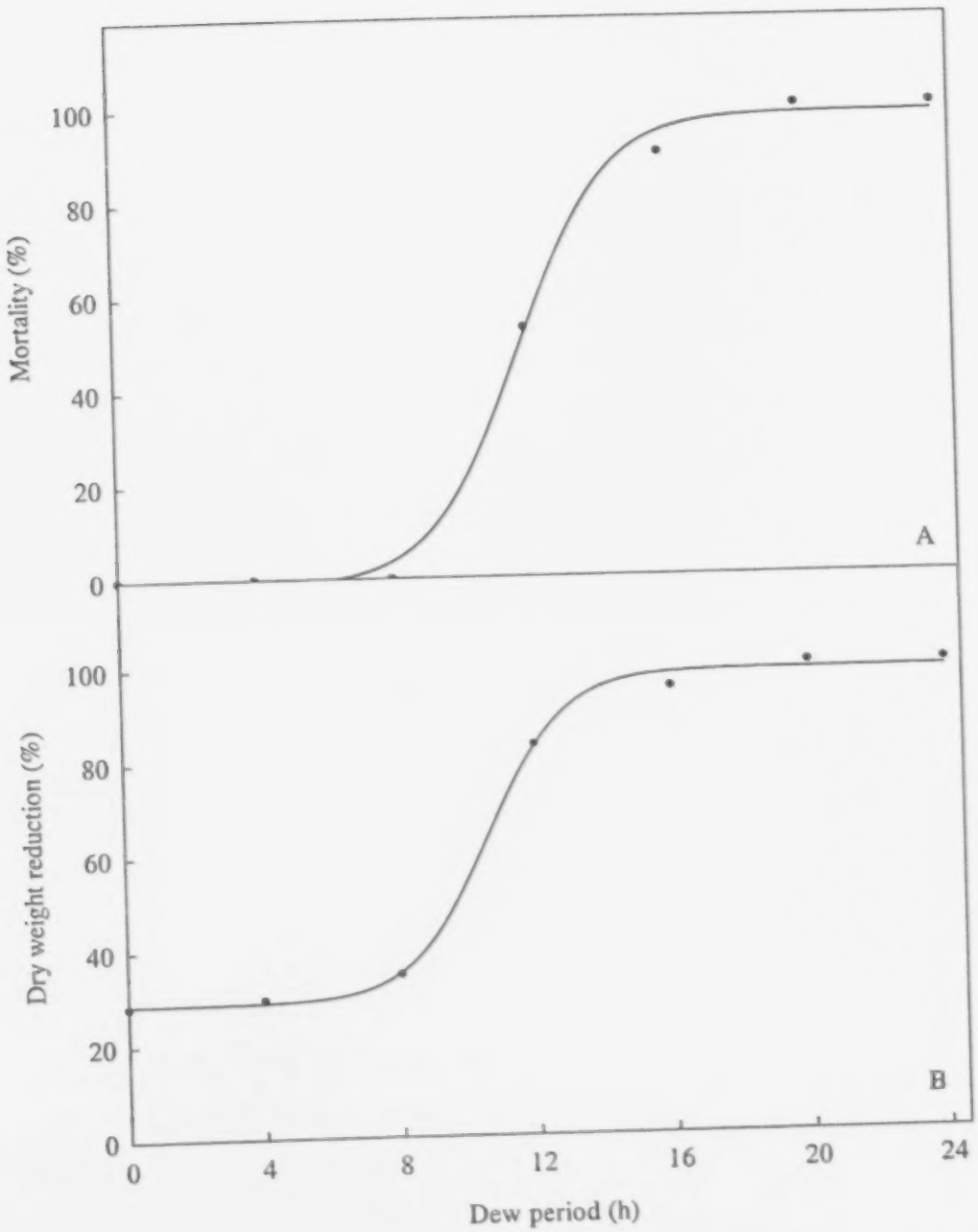

Figure 4.2. Effect of dew period on disease development caused by *Plectosporium tabacinum* on false cleavers, expressed as percent mortality (A) and reduction in dry weight (B) seven days after inoculation. Seedlings of false cleavers at the 1-whorl growth stage were inoculated with a conidial suspension at a concentration of 1 x 10^7 conidia/ml in 1% gelatin solution. Dew temperature was 22 °C (in dark). Data from two trials were not pooled because variances were not homogenous. Data represent the mean of three replicates.

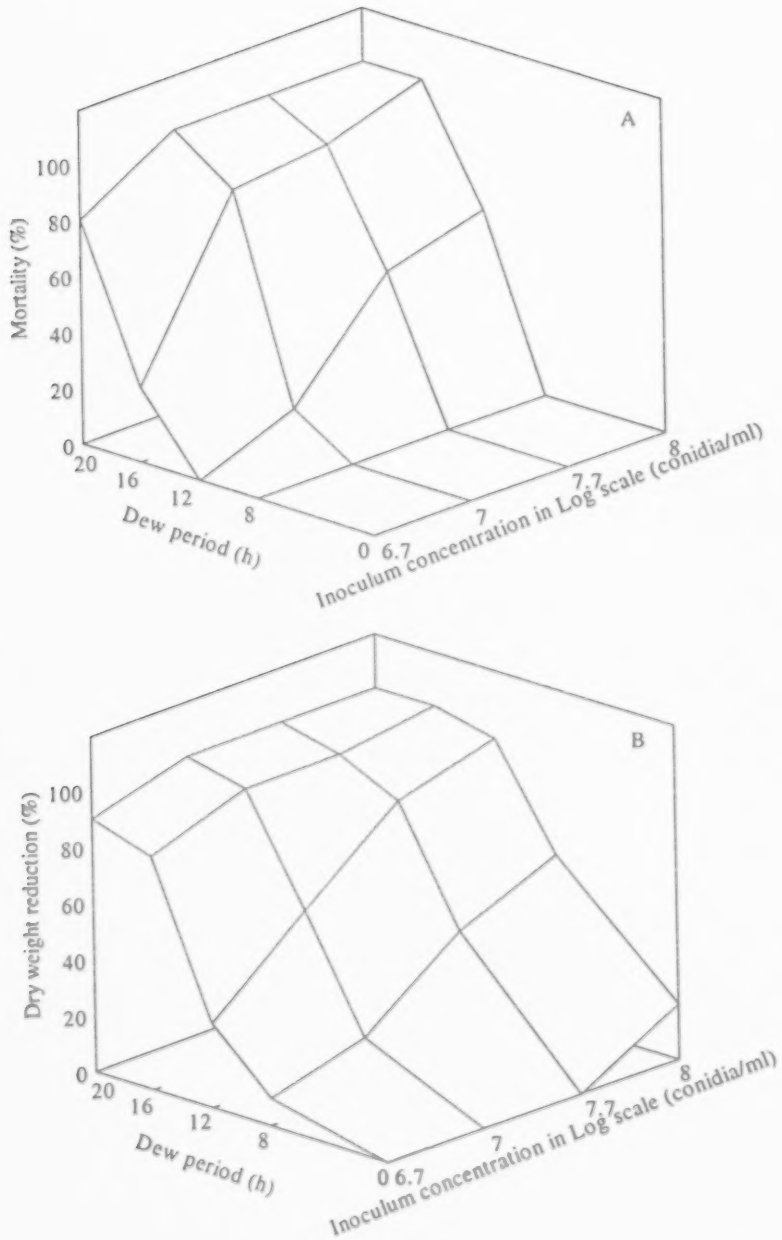

Figure 4.3. Effect of inoculum concentration and dew period duration on disease development caused by *Plectosporium tabacinum* on false cleavers, expressed as percent mortality (A) and reduction in dry weight (B) seven days after inoculation. False cleavers seedlings at the 1-whorl stage were inoculated with conidial suspension at a concentration of $5x10^6$, $1x10^7$, $5x10^7$, or $1x10^8$ conidia/ml in 1% gelatin solution. Dew temperature was 22 °C (in dark) and were placed in the dew chamber for 9, 12, 16, or 20 h and then returned to the greenhouse. Data from two trials were not pooled because variances were not homogenous. Data represent the mean of three replicates.

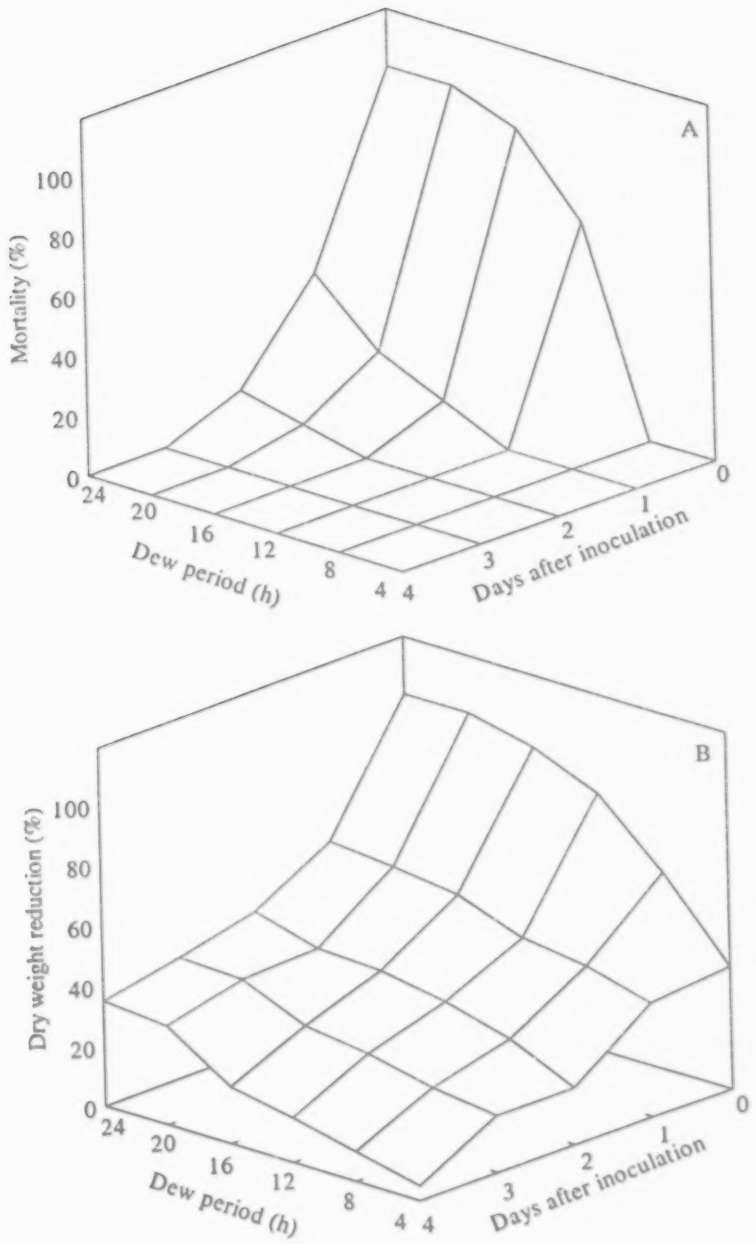

Figure 4.4. Effect of delaying initial dew period on disease development caused by *Plectosporium tabacinum* on false cleavers, expressed as percent mortality (A) and reduction in dry weight (B) seven days after inoculation. Seedlings of false cleavers at the 1-whorl growth stage were inoculated with a conidial suspension at a concentration of 1×10^7 conidia/ml in 1% gelatin solution. Dew temperature was 22 °C (in dark). Data from two trials were not pooled because variances were not homogenous. Data represent the mean of three replicates.

Chapter 5 Field Efficacy of Cleavers Control With *Plectosporium tabacinum*

5.1. Abstract

Field efficacy of *P. tabacinum*, using either spores or culture filtrates as the active ingredient, for control of cleavers was assessed. Results demonstrated that both fungal spores and culture filtrates (containing no living cells) show promise for cleavers control under field conditions. With culture filtrates (containing no living cells), 80-90% cleavers control was observed under field conditions. Few or no phytotoxicity was observed with blank medium control. No damage was observed on wheat seedlings. Less than 5% leaf damage was observed on canola seedlings. However, this damage was completely recovered three weeks after treatment. With the fungal spores, a spore concentration of 1 x 10^7 spores/ml in 0.1% Silwet®, 10% unrefined corn oil, and invert emulsion formulation caused 10%, 50%, and 100% disease severity, respectively. Proper formulations are required for the successful use of *P. tabacinum* spores for cleavers control under field conditions.

5.2. Introduction

The objective of this study is to quantify the efficacy of *P. tabacinum* for the control of chickweed under field conditions.

5.3. Materials and Methods

5.3.1. Plant production.

Seeds of false cleavers (about 200 seeds per pot), canola (about 20 seeds per pot), and wheat (about 15 seeds per pot) were sown in 53-cm diameter peat pot (0.22 m^2) containing field soils (loam, pH 6.7, 42% sand, 40% silt, and 18% clay). The seeded pots were set on an open area and all conditions were similar to actual field conditions. Daily weather conditions during the experimental duration were recoded. After germination, the number of seedlings was counted and the pot was divided into two halves by using a red plastic stripe. About 13-14 days after sown, the majority of false cleavers seedlings were at the cotyledon and 1-whorl stages, with a few plants at the 2-whorl stage.

5.3.2. Spore production.

Sufficient spores were produced using Richard's solution (Tuite, 1969). Two hundred ml of Richard's solution were placed in 500-ml Erlenmeyer flasks and autoclaved for 20 min (100 kPa and 121 °C). After cooling, each flask was inoculated with 'seed' inoculum. Inoculated flasks were incubated on rotary shakers at 150 rpm under ambient laboratory conditions (24 ± 3°C). After 3 d incubation, the culture contents of each flask were chopped using an electric hand blender (Braun Multipractic MR 20, Lunnfield, MA, USA) and passed through four layers of cheesecloth to remove mycelia. The resulting solution was centrifuged at 2,192 g for 15 min to form a conidial pellet (Sorvall RC-5B refrigerated superspeed centrifuge). The supernatant was decanted and the spore pellet was resuspended in an appropriate amount of 1% gelatin (Difco Laboratories, MI, USA) to achieve the desired inoculum concentration as determined with the aid of a haemocytometer. Macerated PDA from solidified plates was added at a rate of 0.02 g ml^{-1} into the resulting 1% gelatin solution as a nutrient supplement.

5.3.3. Culture filtrates preparation.

P. tabacinum culture filtrates were prepared by culturing the fungus in modified Fries medium for 10 days, filtering through 4 layers of cheese cloth to remove the living cells, and concentrating to 1/12 original volume by rotoevaporator.

5.3.4. Efficacy with fungal spores.

Spores of *P. tabacinum* were formulated as an aqueous solution, oil emulsion, or invert emulsion. The aqueous solution consisted of 1 x 10^7 spores/ml plus 0.1% 0.1% Silwet®. The oil emulsion consisted of a 10% oil phase and a 90% water phase. The oil phase consisted of unrefined corn oil. The water phase consisted of the spore suspension, 0.5% macerated solidified PDA, and 0.1% Silwet®. The invert emulsion consisted of a 50% oil phase and a 50% water phase. The oil phase consisted of 70% (v/v) Ultra spray oil, (5% w/v) bee's wax, and (25% w/v) soybean lecithin and the water phase was the same to the oil emulsion. Before spray, both oil and water phases were mixed and blended at high speed in a blender for 45 s. False cleavers seedlings at the cotyledon, 1-whorl, or 2-whorl stage were treated with water (CK), the oil emulsion formulation without *P. tabacinum*

spores (CK), or the oil emulsion formulation of *P. tabacinum* at a density of 5.0 x 10^8 spores/m^2.

5.3.5 Efficacy with culture filtrates.

The concentrated culture filtrates were sprayed to the seedlings of false cleavers, canola, and wheat at two different volumes: 30 ml and 40 ml.

5.3.6. Data analyses.

All experiments were conducted twice in a randomized complete block design with three replications of each treatment. All percentage data were arc sine-transformed before analysis (Gomez and Gomez, 1984). Factorial experiments were analyzed with an analysis of variance considering the effect of each factor individually and their interaction, using PROC ANOVA procedure in SAS (SAS Institute Inc., 1990).

5.4. Results

5.4.1. Efficacy with fungal spores.

Maximum and minimum temperatures (°C), precipitation (mm), sunlight period (h), and average radiation (watts/m^2) during this experiment are shown in the Fig. 7. When fungal spore were formulated in an aqueous solution containing 0.1% Silwet L-77®, disease symptoms were observed on false cleavers seedlings three days after application and about 10% disease severity was observed 14 days after application. When fungal spores were formulated in 10% unrefined corn oil, disease symptoms were observed on false cleavers seedlings two days after application. Disease rating was 50% 14 days after application (Fig. 5.1). *P. tabacinum* spores formulated as an invert emulsion caused 100% disease severity, but the invert emulsion formulation itself also showed severe phytotoxicity to false cleavers (80%). Additional formulation research is required for the successful use of *P. tabacinum* spores for cleavers control under field conditions.

5.4.2. Efficacy with culture filtrates.

Maximum and minimum temperatures (°C), precipitation (mm), sunlight period (h), and average radiation (watts/m^2) during this experiment are shown in the Fig. 5.2. With culture filtrates (containing no living cells), 80-90% cleavers control was observed under field conditions when the culture filtrates were concentrated to 1/12 of the original

volume plus 0.1% Silwet® at an application rate of 30 or 40 ml/m^2 (Fig. 5.3). Little or no phytotoxicity was observed with the medium control. No damage was observed on wheat seedlings. Less than 5% leaf damage was observed on canola seedlings 3-5 days after application and 100% recovery of plants was observed three weeks after treatment.

5.5. Discussions

Additional formulation research is required for the successful use of *P. tabacinum* spores for cleavers control under field conditions. Culture filtrates may provide effective weed control.

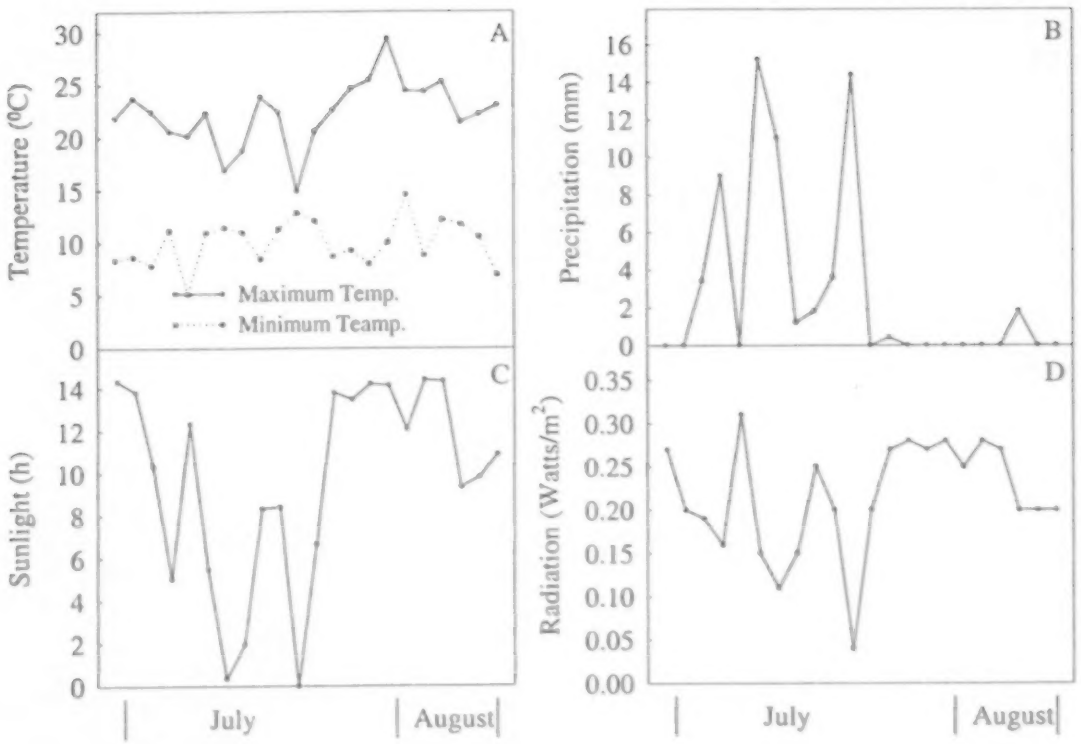

Figure 5.1. Daily weather conditions recorded during the field evaluation of cleavers control with *Plectosporium tabacinum* spores. (A) Maximum and minimum temperatures (°C), (B) Precipitation (mm), (C) Sunlight period (h), and (D) Average radiation (watts/m^2).

Figure 5.2. Control of false cleavers (*Galium spurium*) with *Plectosporium tabacinum* spores under field conditions. A. Treatment with *P. tabacinum* spores (1×10^7 spores/ml) in oil emulsion formulation, and B. Oil emulsion formulation check without *P. tabacinum* spores (CK). The oil emulsion formulation consisted of a 10% oil phase and a 90% water phase. The oil phase consisted of unrefined corn oil. The water phase consisted the spore suspension, 0.5% macerated solidified PDA, and 0.1% Silwet®. Before spray, the two phases were mixed and blended at high speed in a blender for 45 s. False cleavers seedlings at the cotyledon, 1-whorl, or 2-whorl stage were treated with water (CK), the oil emulsion formulation without *P. tabacinum* spores (CK), or the oil emulsion formulation of *P. tabacinum* at a density of 5.0×10^8 spores/m^2.

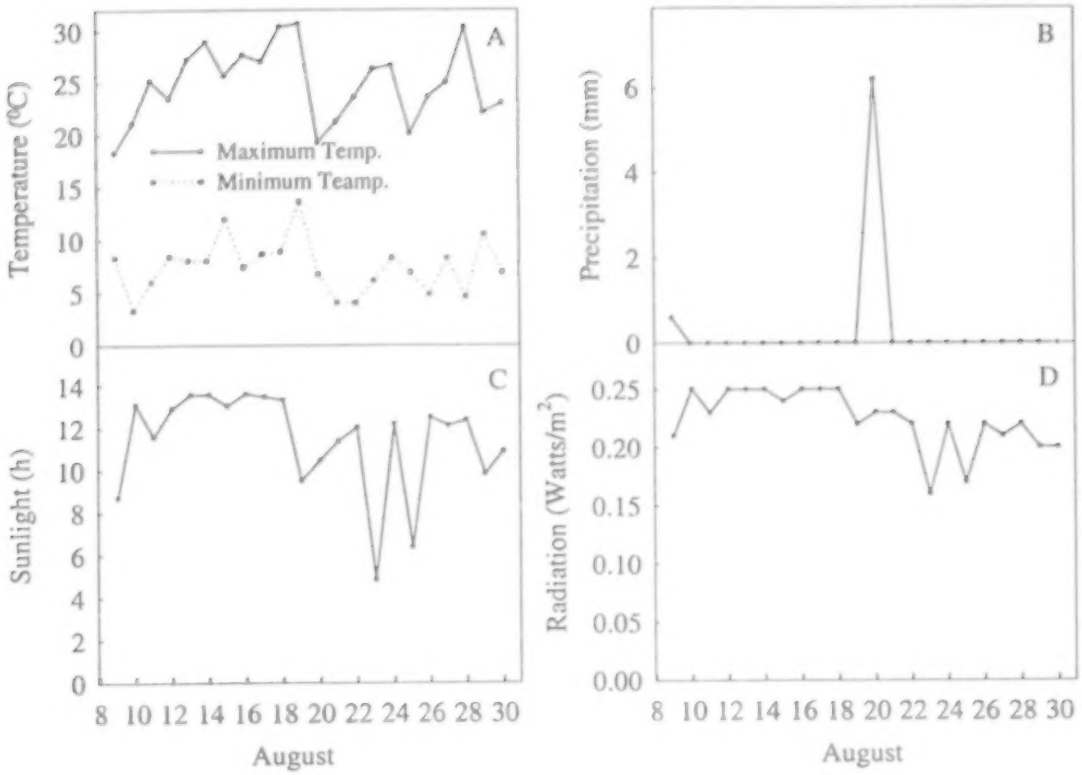

Figure 5.3. Daily weather conditions recorded during the field evaluation of cleavers control with *Plectosporium tabacinum* culture filtrates. (A) Maximum and minimum temperatures (°C), (B) Precipitation (mm), (C) Sunlight period (h), and (D) Average radiation (watts/m^2).

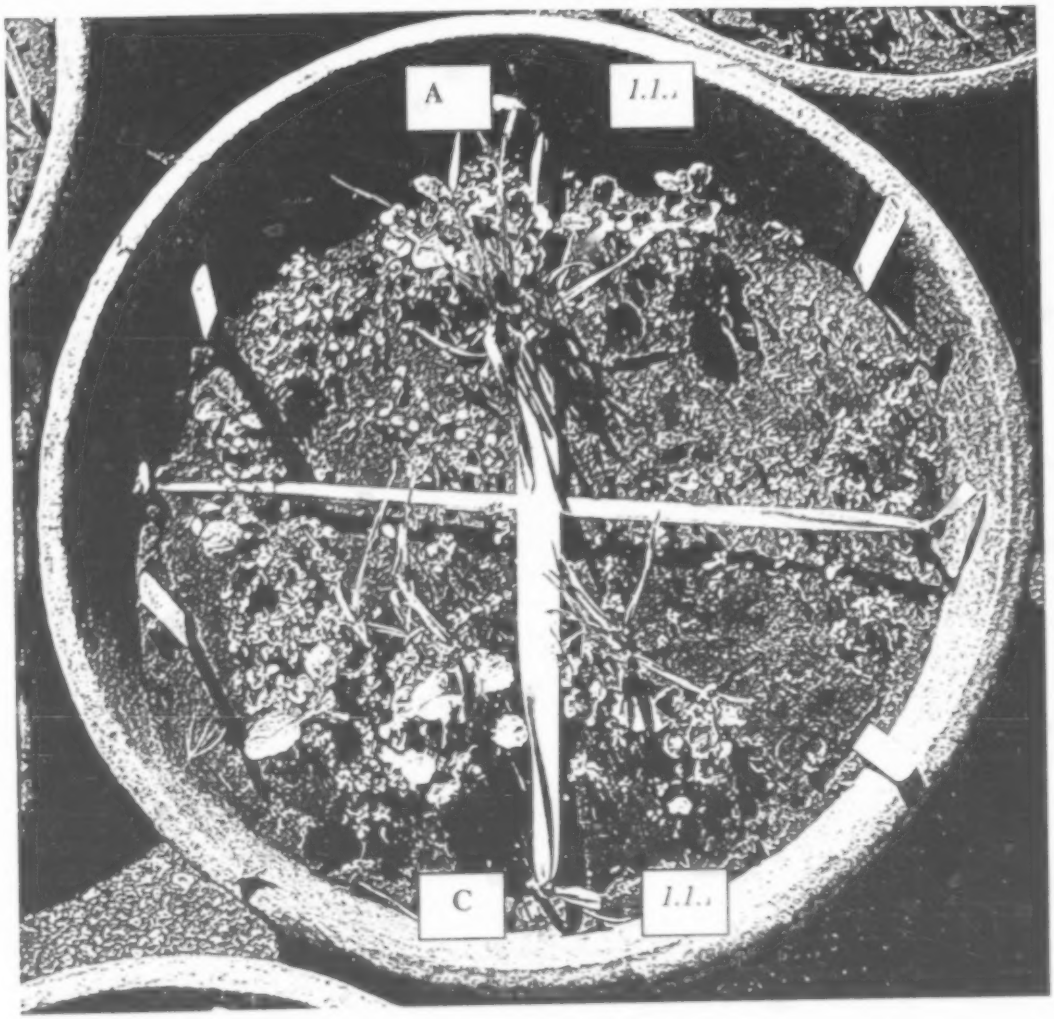

Figure 5.4. Control of false cleavers (*Galium spurium*) with *Plectosporium tabacinum* culture filtrates (containing no living cells) at different application volumes under field conditions. A. Fries medium check without *P. tabacinum* + 0.1% Silwet® at an application volume of 30 ml (CK), B. *P. tabacinum* culture filtrates + 0.1% Silwet® at an application volume of 30 ml, C. Fries Medium check without *P. tabacinum* + 0.1% Silwet® at an application volume of 40 ml (CK), and D. *P. tabacinum* culture filtrates + 0.1% Silwet® at an application volume of 40 ml.

PART II - Chickweed Bioherbicide

SUMMARY

Naturally occurring fungi and bacteria were isolated from various locations across Alberta and from Saskatoon. Of 153 fungal and 448 bacterial isolates, the bacterial isolate, CW00B006C, caused severe disease on chickweed. Typical disease symptoms include chlorosis, resulting in slower chickweed growth and death. Based on phenotypic properties, fatty acid composition, and 16s rDNA sequences, the bacterial strain CW00B006C has been identified as *Burkholderia andropogonis* (Smith 1911) Gillis *et al.* 1995, comb. nov. This bacterium is easily cultured in submerged conditions and its cell production has been characterized using different liquid culture media and various medium pH. Host specificity tests against 36 plant species in 30 genera and 8 families demonstrated that the use of *B. andropogonis* as a bioherbicide for control of chickweed would not cause major concerns to crops and native flora in Canada. However, bioherbicide formu'.tions containing *B. andropogonis* should not be applied to susceptible plants such as corn, chickpea, carnation, and tulip. Various factors impact the herbicidal performance of this bacterium against chickweed. Under greenhouse conditions, increasing the bacterial cell concentration, repeat application, addition of the surfactant Silwet L-77® at 0.1%-0.2%, or application of bacterial inoculum to chickweed seedlings at an older growth stage, significantly increases disease severity on chickweed seedlings. Under field conditions, about 65%-80% disease severity is consistently observed with application of *B. andropogonis* at 10^9-10^{10} CFU/ml plus 0.15% Silwet 77®. A maximum percent dry weight reduction of 79.9% under field conditions was obtained. Moreover, *B. andropogonis* causes equivalent disease severity on Group 2 herbicide resistant chickweed seedlings, providing a new approach for mitigating herbicide resistance development in chickweed. Cell-free culture filtrates cause symptoms similar to those caused by the bacterial cells, indicating the potential of using fermentation broth alone, or combination with bacterial cells, for chickweed control.

Chapter 1. Background Information

Chickweed (*Stellaria media*) is one of the most important weeds in Canada. It is a vigorous competitor for space and nutrients; an aesthetic problem in agricultural crops, market gardens, and urban centers; and listed as a noxious weed in the Noxious Weeds Act of Alberta and Manitoba (Mann et al., 1950; Terkington et al., 1980).

Chickweed is distributed across all Canadian provinces. It is found from Vancouver Island to Newfoundland, and from the United States border to the Mackenzie delta at 69 N (Terkington et al., 1980). Previous data showed that chickweed is more common in B.C. and eastern Canada than in the prairies, however, its populations in the prairie provinces of Canada have been dramatically increasing over the past 10 years. Overall, chickweed relative abundance over these years rose by 4, 6, and 17 rankings in Alberta, Saskatchewan and Manitoba, respectively (Thomas et al., 1998). Presently, chickweed is the most abundant weed species in Alberta, followed by wild oats (Thomas et al., 1997).

The main control method is the use of chemical herbicides that belong mainly to Groups 2 and 4. However, herbicide resistance in chickweed to Group-2 herbicides in Canada and Group-4 herbicides in Europe has been reported (Devine et al., 1991; Hall and Devine, 1990; Lutman & Heath, 1990; O'Donovan et al., 1994). Herbicide resistance development in chickweed substantially limits herbicide choices for its control in most crops and other non-agricultural lands such as home gardens and lawns. This situation provides an opportunity to develop "biological herbicide" or "bioherbicide" or "microbial herbicides" for chickweed control.

The overall goal of this project is the development of effective and environmentally safe bioherbicides for biological control of chickweed. Specific objectives are to:

(1) select bioherbicide candidates for the control of chickweed,

(2) develop techniques to produce propagules of the bioherbicide candidates,

(3) determine optimum conditions and limiting factors for maximum effectiveness of the bioherbicide candidates, and

(4) quantify the field efficacy.

Literature Cited

1. Devine, M.D., Marles, M.A.S. and Hall, L.M. 1991. Inhibition of acetolactate synthase is susceptible and resistant biotypes of *Stellaria media*. Pestic. Sci. 31: 273-280.
2. Hall, L.M. and Devine, M.D. 1990. Cross-resistance of a chlorsulfuron-resistant biotype of *Stellaria media* to a Triazolopyrimidine herbicide. *Plant Physiol.* 93: 962-966.
3. Lutman and Heath, 1990. Variations in the resistance of *Stellaria media* to mecoprop due to biotype, application method and 1-aminobenzotriazole. Weed Res. 30:129-137.
4. Mann, H.H. and Barners, T.W. 1950. The competition between barley and certain weeds under controlled conditions. 4. Competition with *Stellaria media*. Ann. Appl. Biol. 37:139-148.
5. O'Donovan, J.T., Jeffers, G.M., Maurice, D., and Sharma, M.P. 1994. Investigation of a chlorsulfuron-resistant chickweed weed [*Stellaria media* (L.) Vill.] population. Can. J. Plant Sci. 74: 693-697.
6. Thomas, A.G., Frick, B.L., and Hall, L., 1997. Weed population shifts in Alberta. Proceedings, 1997 ECW Meetings, Charlottetown, PEI.
7. Thomas, A.G., Frick, B.L., Juras, L.T, Hall, L., van Acker, R., and Joose, D. 1998. Changes in weed distributions indicated by quantitative surveys in the prairie provinces of Canada over 10 years. Proceedings of the 38th Annual WSSA Conference, Chicago, IL.
8. Turkington, R., Kenkel, N.C., and Krankko, G.D. 1980. The biology of Canadian weeds. 42. *Stellaria media* (L.) Vill. *Can. J. Plant Sci.* 60:981-992.

Chapter 2 Collection and Screening of Bioherbicide Candidates for Control of Chickweed

2.1. Abstract

Naturally occurring fungi and bacteria were isolated from various locations across Alberta and from Saskatoon. A total of 52 fungal and 25 bacterial isolates with some pathogenicity to chickweed were found after the demonstration of Koch's postulates. Of these isolates, the bacterial isolate, CW00B006C, typically causes disease symptom on chickweed of chlorosis and necrosis, resulting in slower plant growth and death. CW00B006C was selected as the bioherbicide candidate for further study. The use of this bacterium as a bioherbicide does not require dew or free moisture to cause the disease.

2.2 Introduction

The use of living microorganisms, mainly plant pathogens, for control of weeds has received increasing interest in the last three decades, especially the bioherbicide approach (Charudattan, 2001). The bioherbicide approach involves three major phases or stages: 1) discovery, 2) development, and 3) deployment (Templeton, 1982). Major activities in the discovery phase involve the collection of diseased plant material, the isolation of causal organisms, and the demonstration of Koch's postulates (Watson, 1993). To date, there has been no research to extensively search for chickweed diseases with an aim to develop a bioherbicide.

Unlike the classical biological control approach, the bioherbicide approach usually relies on the use of endemic pathogens (Watson, 1993). In Canada, three fungi, *Septoria stellariae* Ro. & Desm., *Melampsorella caryophyllacearum* Schroet., and *Puccinia arenariae* (Schum.) Wint., were associated with chickweed (Conners, 1967; Tom, 1964). But it is unknown whether these fungi can be isolated and used as bioherbicide candidates. In addition, the existence of other fungi and bacteria that infect chickweed and possess potential as bioherbicides remains unknown.

Thus, the objectives of this study are to (1) collect diseased chickweed plants, (2) isolate the causal microorganism with focus on fungi and bacteria, and (3) demonstrate the Koch's postulates to identify the pathogenic fungi and bacteria with bioherbicide potential.

2.3. Materials And Methods

2.3.1. Collection and isolation of fungi and bacteria

2.3.1.1. Collection of diseased chickweed.

Several field trips were made from May to September in 1999 and 2000 to collect diseased chickweed plant materials from agricultural crops and market gardens in the districts of Vegreville, Tofield, Busby, Ryley, Ranfurly, Fort Assiniboine, Lamont, Spruce Grove, Leduc, Red Deer, Calgary, Edmonton, and Saskatoon. Diseased plant parts of chickweed were collected, cut to appropriate size, and transported at 4 °C.

2.3.1.2. Isolation of causal organisms.

Isolation was carried out in the lab within 24 h of field collection in order to keep the samples as fresh as possible. Diseased plant material was sorted based on whether fungal or bacterial disease was most likely to have caused disease symptoms. To isolate fungi, leaf, stem or root pieces with lesions were surface sterilized with 0.5% sodium hypochlorite solution and incubated on fresh potato dextrose agar (PDA; Difco, Detroit, MI). Fungi that grew from the lesions were isolated. For all isolated fungi, single cell colonies were obtained by using standard single spore or hyphal tip techniques to ensure species purity (Tuite, 1969). To isolate bacteria, pieces of leaves or stems with lesions were washed with running tap water, placed into a drop of sterile distilled water, and macerated. A loopfull of the macerated solution was streaked across a plate of nutrient agar, nutrient glucose agar, and nutrient broth yeast extract agar (Schaad, 1988). Single, morphologically distinct bacterial colonies were then selected from culture plates after 4-7 days and streaked on a fresh plate of culture media to obtain pure cultures.

2.3.2. Maintenance of microorganisms.

A 15% glycerol solution was prepared as follows: Add 15 ml glycerol to 85 ml of distilled water, autoclave and cool to room temperature. Then, 1.8 ml of 15% glycerol solution was dispensed into 2 ml cryovials. A pure culture of fungi or bacteria was scraped with a scalpel blade and placed in each of 3 cryovials of glycerol solution. Cryovials of samples were placed in a Mr. Frosty (Nalgene Cryo Freezing container, Cat No 5100-0001) in a freezer at –80 °C for a minimum time of 85 minutes to freeze at a

rate of 1 °C per minute to –80 °C. Frozen samples were then placed in racks for storage at –80 °C as stock cultures.

2.3.3. Screening of fungi and bacteria

2.3.3.1. Inoculum preparation

For fungal pathogens, a cryovial of stock culture was warmed to room temperature in a 36°C water bath and the contents were dispensed in 200 µl aliquots on the surface of potato dextrose agar (PDA) plates, and spread with a sterile glass rod. The PDA plate was incubated for 5 days at 22 °C under 12 h light provided by two, 20W, 60 cm long cool white fluorescent tubes placed 30 cm above the PDA plates. Single-spore colonies were then made for all fungal isolates using standard single-spore-technique and cultures were incubated under conditions as described above. Sufficient spores for experiments were produced by transferring spores from a single-cell colony onto several PDA plates (spread plates). Plates were incubated as above for 1-2 wks. Spores were harvested by gently scraping the surface of the agar and transferring into sterilized distilled water. Spore concentrations were determined using a haemocytometer.

For bacterial pathogens, a cryovial of stock culture was warmed to room temperature in a 36°C water bath. A 50 µl aliquot of suspension from the vial was added to each 18 x 150 mm glass test tube containing 3 ml nutrient glucose broth (NGB) at a pH of 6.8. The test tubes were incubated on an orbit shaker at 200 rpm for 24-72 h under ambient laboratory conditions (24 °C ± 3). Contents of tubes were used as 'seed inoculum'. The bacterial culture for inoculations was produced by placing 75 ml of NGB (pH 6.8) in each 250-ml Erlenmeyer flasks, autoclaving, cooling, inoculating with 1 ml per flask of the 'seed inoculum', and incubating flasks on an orbit shaker at 200 rpm for 24-72 h under ambient laboratory conditions (24 °C ± 3). About 30 ml of cell suspension from each flask was then placed in a 50 ml centrifuge tube and centrifuged 10 minutes at 3700 rcf (Sorvall RC-5B refrigerated superspeed centrifuge). Supernatant was poured from each tube and the bacterial pellet was resuspended in 20 ml of 0.01 M, pH 7 phosphate buffer. Viable bacterial cell production was determined using the dilution plate count method. Serial dilutions (10x) were performed in dilution tubes containing 9 ml of sterile 0.01 M phosphate buffer, pH 7, and 100 µl of three appropriate dilutions were spread plated on nutrient glucose agar (NGA). Plates were incubated under ambient

laboratory conditions for about 4 days. Colony forming units (CFU) per ml were determined by counting colonies on plates with 10-200 CFU per plate.

2.3.3.2. Plant production

Fungal or bacterial virulence against chickweed was assessed using a single batch of chickweed seeds for all experiments. Seeds were sown in 10-cm-diameter pots filled with pre-prepared soil mix. The soil mix consisted of 140 L soil (loam, pH 6.7, 42% sand, 40% silt, and 18% clay), 107 L sand, 160 L Sunshine Growing Mix (SunGro Horticulture, Bellevue, Washington), 62 L Fibrous Blond Shagnum Peat Moss (Premier Pro Moss, Riviere-du-Loup, Quebec), 113 L vermiculite (Therm-O-Rock, Chandler, Arizona), 230 g dolomite lime, and 150 g Super Phosphate (0-45-0) per batch of soil. Seeded pots were placed in a greenhouse with 23/20 ± 4°C day/night temperature, a 16 h photoperiod, an average light intensity of 300 $\mu Em^{-2}s^{-1}$, and an average relative humidity of 45-50%. After germination, seedlings were thinned to five plants per pot.

2.3.3.3. Inoculation procedure

For fungal pathogens, chickweed seedlings at the 4- to 6-leaf stage were inoculated with 5×10^5 to 5×10^6 spores/ml to run-off with 0.05% gelatin as a wetting agent, using an airbrush at 100 kPa. Unless otherwise indicated, after spraying, pots were placed in a dark dew chamber with 100% relative humidity at 22 °C for 48h. Subsequently, pots were transferred back to the greenhouse with conditions as mentioned above. Control treatments were sprayed with distilled water containing only the wetting agent.

For bacterial pathogens, chickweed seedlings at the 4- to 6-leaf stage were inoculated with 10^7-10^{10} CFU/ml of bacterial cells suspended in 0.01 M, pH 7 phosphate buffer plus 0.1% Silwet L-77® (wetting agent) using a airbrush at 100 kPa until all leaf surfaces were evenly wet. Immediately after spraying, pots were returned to the greenhouse. Control treatments were sprayed with 0.01 M phosphate buffer containing only the wetting agent.

2.3.3.4. Pathogenicity and virulence to chickweed

Fungal or bacterial virulence on chickweed were assessed 7 and 14 days after inoculation using a 0 to 3 scale (0 – no symptom, 1 – light infection, 2 – moderate infection, and 3 –

severe infection to death). For each isolate tested, there were three replications arranged in a completely randomized design. The experiments were repeated at least once.

2.3.4. Disease symptoms of CW00B006C in chickweed

To characterize the typical symptoms associated with chickweed disease caused by isolate CW00B006C, chickweed plants inoculated with the bacterium in various greenhouse and field experiments were observed from the onset of symptoms for a three wk period after spraying. Symptoms from the various experiments were recorded, compared, and summarized.

2.4. Results And Discussions

2.4.1. Collection and isolation of fungi and bacteria

From 13 different locations, a total of 153 fungal isolates and 448 bacterial strains were isolated from chickweed and stored at –80 °C (Table 2.1). The findings in this study demonstrated that endemic fungi and bacteria associated with chickweed are much more common than previously reported (Conners, 1967; Toms, 1964). These naturally occurring fungi and bacteria provided a sound base for searching for a bioherbicide candidate.

2.4.2. Screening of fungi and bacteria.

A total of 52 fungal and 25 bacterial isolates with some pathogenicity to chickweed were found (Table 2.2 and Table 2.3). Of these isolates, only three fungi and one bacterium are highly pathogenic to chickweed and thus deserve consideration as bioherbicide candidates.

Fungal isolate CW98-235 was the most virulent of the fungal isolates (data not shown). All three fungi, however, required a prolonged dew period (48 h) to cause infection, while the bacterium required no free moisture and had excellent virulence against chickweed. Thus, the bacterial isolate, CW00B006C, was selected as the bioherbicide candidate for further study.

2.4.3. Disease symptoms of CW00B006C in chickweed

Isolate CW00B006C infection of chickweed causes chlorosis on treated leaves and stems, sometimes with necrotic lesions, within 5-7 days after bacterial treatment (Figure 2.1). As the disease progress, plant growth dramatically slows down. Existing leaves and stem senesce. Emerging leaves were chlorotic and small. Severe infections caused plant death.

Bacteria have excellent potential as weed biocontrol agents because they can be delivered into the xylem via stomata and other natural plant openings with the aid of a new type of surfactant - nonionic organosilicone surfactants (e.g. Silwet-77®) (Zidack et al., 1992; Johnson et al., 1996). As a result, the bacteria do not require a prolonged dew period or free moisture for its infection and colonization that rarely happy to meet under the field conditions, especially in western Canada. This is a significant advantage over the use of a fungus as a bioherbicide. Therefore, the bacterial isolate CW00B006C possesses great potential as a bioherbicide.

2.5. Literature Cited

1. Charudattan, R. 2001. Biological control of weeds by means of plant pathogens: significance for integrated weed management in modern agro-ecology. Biocontrol 46:229-260.
2. Conners, I.L. 1967. An annotated index of plant diseases in Canada and fungi recorded on plants in Alaska, Canada and Greenland. Can. Dep. Agric. Publ. 1251. Ottawa, Ont. pp381.
3. Johnson, D.R., Wyse, D.L. and Jones, K.J. 1996. Controlling weeds with phytopathogenic bacteria. Weed Technology 10:621-624.
4. Templeton, G.E. 1982. Biological herbicides: discovery, development, deployment. Weed Sci.:30:430-433.
5. Toms, H.N. 1964. Plant diseases of Southern British Columbia – A host index. Can. Plant Dis. Surv. 44:143-225. Res. Br., Can. Dep. Agric., Ottawa, Ont.
6. Watson, A.K. 1993. Biological Control of Weeds Handbook. Weed Science Society of American, Champaign, Ill., USA.
7. Zidack, N.K., Backman, P.A., and Shaw, J.J. 1992. Promotion of bacterial infection of leaves by an organosilicone surfactant: implications for biological weed control. Biological Control 2:111-117.

Table 2.1 Number of fungi and bacteria isolated from chickweed at various locations

Location	No. of Isolates	
	Fungi	Bacterial
Vegreville	35	10
Tofield	11	21
Busby	10	34
Ryley	1	6
Ranfurly	46	-
Fort Assiniboine	2	47
Lamont	6	-
Spruce Grove	9	95
Leduc	1	10
Red Deer	-	206
Calgary	-	16
Saskatoon	32	3

Table 2.2 Virulence of fungal isolates on chickweed (*Stellaria media*)*

Isolates tested	153
Non-pathogenic isolates	101
Pathogenic isolates	
Slight infection (+)	46
Moderate infection (++)	3
Severe infection (+++)	3
Bioherbicide prospects	1

* Seedlings of chickweed at the 4- to 6-leaf stage were inoculated with 10^5 to 10^6 spores/ml, placed in a dew chamber at 22 °C for 48 h and subsequently maintained in a greenhouse. Pathogenicity and virulence was rated 7 days after inoculation using a 0 to 3 grading system where 0 = no infection, + = light infection, ++ = moderate infection, and +++ = severe infection to death.

Table 2.3 Virulence of bacterial isolates on chickweed (*Stellaria media*)*

Isolates tested	448
Non-pathogenic isolates	382
Pathogenic isolates	
Slight infection (+)	58
Moderate infection (++)	7
Severe infection (+++)	1
Bioherbicide prospects	1

* Seedlings of chickweed at the 4- to 6-leaf stage were inoculated with 10^7 to 10^{10} CFU/ml and subsequently maintained in a greenhouse. Pathogenicity and virulence was rated 14 days after inoculation using a 0 to 3 grading system where 0 = no infection, + = light infection, ++ = moderate infection, and +++ = severe infection to death.

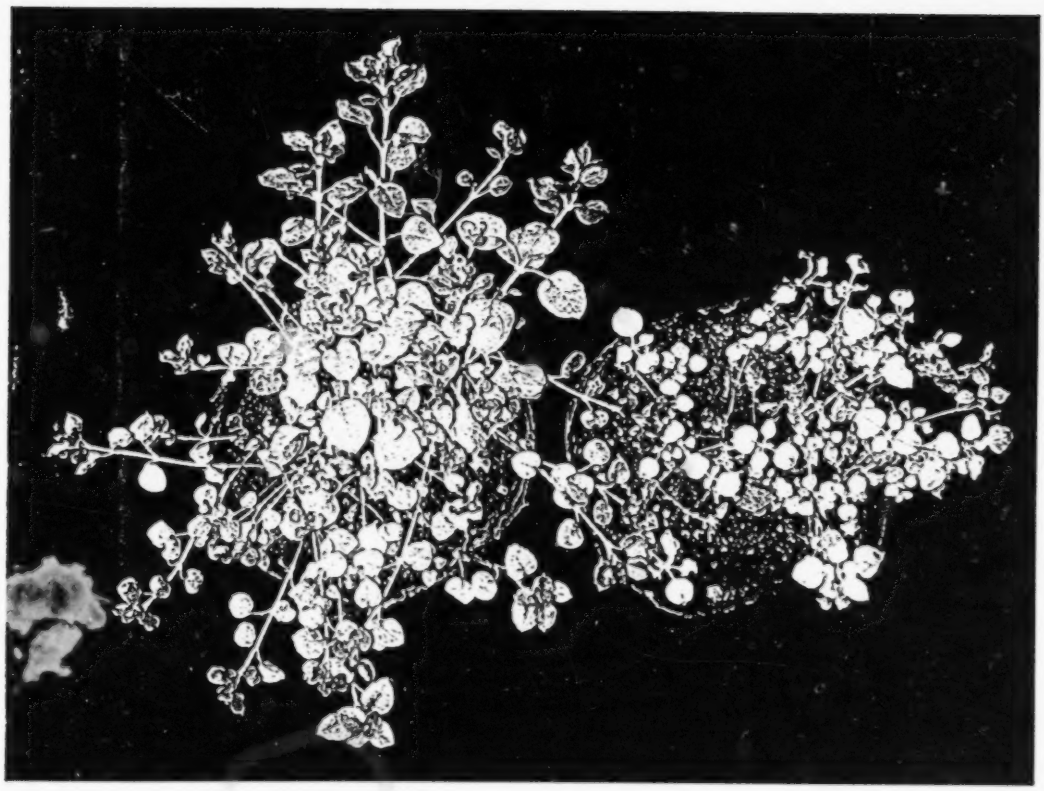

Figure 2.1 Disease symptoms caused by the bacterial isolate CW00B006C in chickweed.

Chapter 3 Characterization and Identification of Bacterium CW00B006C

3.1. Abstract

Based on phenotypic properties, fatty acid composition, and 16s rDNA sequences, the bacterial strain CW00B006C has been identified as *Burkholderia andropogonis* (Smith 1911) Gillis et al. 1995, comb. nov. This is the first report that *B. andropogonis* infects chickweed. Further development of *B. andropogonis* as a bioherbicide for chickweed control is well justified.

3.2. Introduction

The native bacterium, strain CW00B006C, caused severe disease on chickweed. In order to further evaluate and develop this bacterium as a bioherbicide, its identification is essential to provide information on its pathogenicity, culture conditions, and phylogenetic relations. Various techniques are available for bacterial identification including phenotypic and genotypic analyses (Bagsic-Opulencia et al., 2001; Viallard et al., 1998; Whitford et al., 2001). The objective of this study was to identify this bacterium using those available technologies including physiological and biochemical characterization, fatty acid composition, and 16s rDNA sequence. In addition, the bacterial growth and diseases symptoms were also characterized.

3.3. Materials and Methods

3.3.1. Phenotypic analyses

The bacterial isolate CW00B006C was characterized and identified based on phenotypic properties including physiological and biochemical characterization and fatty acid composition.

3.3.1.1.Physiological and biochemical characterization

Characterization included gram staining, motility, carbon substrate assimilation, oxidase and other physiological activities using previously described methods (Coeyne et al. 1999; Viallard et al.1998; Hu et al. 1991). Carbon substrate assimilation tests were performed using auxanographic API 50CH strips (bioMerieux) as recommended by the manufacturer.

3.3.1.2. Fatty acid composition (MIDI-FAME)

The bacterial isolate CW00B006C was grown overnight at 27° C on trypticase soy agar and harvested for extraction of total cellular fatty acids. Total cellular fatty acids were extracted and methylated for fatty acid methyl ester (FAME) analysis by gas chromatography (Paisley 1996; Vandamme *et al.* 1992) and the profiles compared with a computerized database using Sherlock microbial id ntification system software (MIDI, Newark, DE).

3.3.2. Genotypic analyses (16S rDNA sequence)

Genomic DNA was prepared from bacterial isolate CW00B006C as described by Whitford *et al.* (1998). 16S ribosomal rRNA genes (rDNA) were amplified using primers FP1 (5'AGA GTT YGA TYC TGG CT-3') and R1492 (5'-TAC GGY TAC CTT GTT ACG ACT-3') based on primers described by Lane (1991). Primers and PCR reaction conditions have been previously described (Whitford *et al.*, 2001). The 16S rDNA PCR products were purified using a QIAquick PCR purification kit (QIAGEN) and the recovered PCR products were quantified using a DyNAQuant 200 Fluorometer (Hoefer). The PCR products were directly sequenced using a Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech) using IRD800-labeled M13 forward and reverse primers (LI-COR Inc., Lincoln, Nebraska), plus the IRD800-labeled 16S rDNA specific primers FP1, EUB338f (5'-ACT CCT ACG GCA GGC AG-3'), 519r (5'-GWA TTA CCG CGG CKG CTG-3'), 926f (5'-AAA CTY AAA KGA ATT GAC GG-3'), 1100r (5'-AGG GTT GCG CTC GTT G-3'), and 1492r. Sequence products were analyzed on a LI-COR model 4000L sequencer. Sequence fragments were assembled and edited using Sequencher version 3.1 (Gene Codes Corp., Ann Arbor, MI). The resulting sequence was analyzed using the Similarity Matrix Program at the Ribosomal Database Project II (RDPII; rdp.cme.msu.edu/html/).

3.4. Results and Discussions

3.4.1. Phenotypic analyses

3.4.1.1. Physiological and biochemical characterization

Isolate CW00B006C is a motile, gram negative rod, 0.5 x 1.5-2.5 µm, that grows only aerobically without production of any soluble or fluorescent pigments. The isolate has

urease activity, utilizes citrate and produces poly-β-hydroxybutyrate. The isolate does not produce indole or have oxidase, arginine and lysine dihydrolase, gelatin liquification, lecithinase, β-galactosidase and β-glucouronidase activities. The isolate grows well at 30 °C but not at 37 °C or higher. Tests based on the assimilation of 49 carbon sources exhibited that isolate CW00B006C utilizes 20 carbons as a sole carbon source (Table 3.1). Isolate CW00B006C matches closely to *Burkholderia andropogonis* (Smith 1911) Gillis et al. 1995, comb. Nov., differing in its utilization of D-xylose but not gluconate (Hu et al. 1991; Gillis et al., 1995; Viallard et al. 1998).

3.4.1.2. Fatty acid composition

The main fatty acids (>1%) in the cells of strain CW00B006C are 14:0 (6.1%), 16:0 (14.2%), 18:1ω7c (22.4%), 16:1 2-OH (4.6%), 16:0 2-OH (5.7%), 16:0 3-OH (4.5%), 18:1 2-OH (2.2%), 17:0 cyclo (5.2%), 19:0 cyclo ω8c (7.1%), summed feature 2 (14:0 3-OH; 4.5%) and summed feature 3 (16:1ω7c; 21.5%). These results match published values for *B. andropogonis* (Coeyne et al., 2001a; Stead, 1992). In particular, the presence of 14:0 3-OH, 16:1 2-OH, 16:0 2-OH and 16:0 3-OH and no other hydroxy fatty acids are indicative of *Burkholderia* spp. (Stead, 1992). Further, the predominance of 16:0, 18:1ω7c and summed feature 3 are indicative of *B. andropogonis* (Coeyne et al., 2001a; Coeyne et al., 2001b; Coeyne et al., 2001c; Vandamme et al., 1997; Vandamme et al., 2000; Zhang et al., 2000).

3.4.2. Genotypic analyses (16S rDNA sequencing)

A 1408 base pair sequencing product was produced from amplification of the rDNA from isolate CW00B006C. The 16S rDNA sequence of isolate CW00B006C is very similar to that of *Burkholderia andropogonis* ATCC 19311 (formerly *Pseudomonas woodsii*; similarity value 98.6%) and *Burkholderia andropogonis* ATCC 23061^T. Therefore, genotypic analyses confirmed the identification of isolate CW00B006C as *B. andropogonis* (Smith 1911) Gillis *et al.* 1995, comb. nov.

This is the first report that *B. andropogonis* infects chickweed. *B. andropogonis* has never been studied as a biocontrol agent for control of chickweed. It is well justified to further develop *B. andropogonis* as a biocontrol agent for control of chickweed.

3.5 Literature Cited

1. Bagsic-Opulencia, R.D., A.C. Hayward and M. Fegan. 2001. Use of ribotyping and random amplified polymorphic DNA to differentiate isolates of *Burkholderia andropogonis*. J. Appl. Microbiol. 91: 686-696.
2. Coenye, T., B. Holmes, K. Kersters, J.R.W. Govan and P. Vandamme. 1999. *Burkholderia cocovenenans* (van Damme et al. 1960) Gillis et al. 1995 and *Burkholderia vandii* Urakami et al. 1994 are junior synonyms of *Burkholderia gladioli* (Severini 1913) Yabuuchi et al.1993 and *Burkholderia plantarii* (Azegami et al. 1987) Urakami et al. 1994, respectively. Int. J. Syst. Bacteriol. 49: 37-42.
3. Coenye, T., S. Laevens, M. Gillis and P Vandamme. 2001a. Genotypic and chemotaxonomic evidence for the reclassification of *Pseudomonas woodsii* (Smith 1911) Stevens 1925 as *Burkholderia andropogonis* (Smith 1911) Gillis et al. 1995. Int. J. Syst. Evol. Microbiol. 51: 183-185.
4. Coenye, T., S. Laevens, A. Willems, M. Ohln, W. Hannant, J.R.W. Govan, M. Gillis, E. Falsen and P. Vandamme. 2001b. *Burkholderia fungorum* sp. nov. and *Burkholderia caledonica* sp. nov., two new species isolated from the environment, animals and human clinical samples. Int. J. Syst. Evol. Microbiol. 51: 1099-1107.
5. Coenye, T., E. Mahenthiralingam, D. Henry, J.J. LiPuma, S. Laevens, M. Gillis, D.P. Speert and P. Vandamme. 2001c. *Burkholderia ambifaria* sp. nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolates. Int. J. Syst. Evol Microbiol. 51: 1481-1490.
6. Gillis, M., T.V. Van, R. Bardin, M. Goor, P. Hebbar, A. Willems. P. Segers, K. Kersters, T. Heulin and M.F. Fernandez. 1995. Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N2-fixing isolates from rice in Vietnam. Int. J. Syst. Bacteriol. 45: 274-289.
7. Hu, F.-P., J.M. Young and C.M. Triggs. 1991. Numerical analysis and determinative tests for nonfluorescent plant-pathogenic *Pseudomonas* spp. and genomic analysis and reclassification of species related to *Pseudomonas avanae*. Int. J. Syst. Bacteriol. 41: 516-525.

8. Lane, D.J. 1991. 16S/23S rRNA sequencing. In E. Stackebrandt, Goodfellow, M. (ed.) Nucleic Acid Techniques in Bacterial Systematics, pp 115-175. John Wiley and Sons, New York.

9. Paisley, R. 1996. MIS whole cell fatty acid analysis by gas chromatography: training manual. MIDI, Newark, DE.

10. Stead, D.E. 1992. Grouping of plant-pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. Int. J. Syst. Bacteriol. 42: 281-295.

11. Vandamme, P., B. Holmes, M. Vancanneyt, T. Coenye, B. Hoste, R. Coopman, H. Revets, S. Lauwers, M. Gillis, K. Kertsers and J.R.W. Govan. 1997. Occurrence of multiple genomovars of *Burkholderia cepacia* in cyctic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. Int. J. Syst. Bacteriol. 47: 1188-1200.

12. Vandamme, P., E. Mahenthiralingam, B. Holmes, T. Coenye, B. Hoste, P. de Vos, D. Henry and D.P. Speert. 2000. Identification and population structure of *Burkholderia stabilis* sp. nov. (formerly *Burkholderia cepacia* Genomovar IV). J. Clin. Microbiol. 38: 1042-1047.

13. Vandamme, P., M. Vancanneyt, B. Pot, L. Mels, B. Hoste, D. Detwettinck, L. Vlaes, C. van den Borre, R. Higgins, J. Hommez, K. Kersters, J.-P. Butzler and H. Goossens. 1992. Polyphasic taxonomic study of the emended genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an aerotolerant bacterium isolated from veterinary specimens. Int. J. Syst. Bacteriol. 42: 344-356.

14. Viallard, V., I. Poirier, B. Cournoyer, J. Haurat, S. Wiebkin, K. Ophel-Keller and J. Balandreau. 1998. *Burkholderia graminis* sp. nov., a rhizospheric *Burkholderia* species, and reassessment of [*Pseudomonas*] *phenazinium*, [*Pseudomonas*] *pyrrocinia* and [*Pseudomonas*] *glathei* as *Burkholderia*. Int. J. Syst. Bacteriol. 48: 549-563.

15. Whitford, M.F.; R.J. Forster, C.E. Beard, J. Gong and R.M. Teather. 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. Anaerobe 4: 153-163.

16. Whitford, M.F.; M.A. McPherson, R.J. Forster and R.M. Teather. 2001. Identification of bacteriocin-like inhibitors from rumen *Streptococcus* spp. and isolation and characterization of Bovicin 255. Appl. Environ. Microbiol. 67: 569-574.

17. Zhang, H., S. Hanada, T. Shigematsu, K. Shibuya, Y. Kamagata, T. Kanagawa and R. Kurane. 2000. *Burkholderia kuruiensis* sp. nov., a trichloroethylene (TCE)-degrading

bacterium isolated from an aquifer polluted with TCE. Int. J. Syst. Evol. Microbiol. 50: 743-749.

Table 3.1 Carbon assimilation of CW00B006C as opposed to type strain of *Burkholderia andropogonis*[*]

	CW00B006C	*Burkholderia andropogonis*
Glycerol	+	+
D-arabinose	+	+
L-arabinose	+	+
Ribose	+	+
D-xylose	+	-
Adonitol	+	+
Galactose	+	+
D-glucose	+	+
D-fructose	+	+
D-mannose	+	+
Rhamnose	+	V
Inositol	+	+
Mannitol	+	+
Sorbitol	+	+
Lactose	+	+
Trehalose	+	V
D-raffinose	+	V
D-lyxose	+	+
D-fucose	+	V
D-arabitol	+	+
Gluconate	-	+
Erythitol	-	-
L-xylose	-	-
β-methyl-xyloside	-	-
L-sorbose	-	-
Dulcitol	-	-
α-methyl-D-mannoside	-	-
α-methyl-D-glucoside	-	-
N-acetyl-glucosamine	-	-
Amygdalin	-	-
Arbutine	-	-
Aesculin hydrolysis	-	-
Salicin	-	-
Cellobiose	V	-
Maltose	V	-
Melibiose	-	-
Sucrose	-	-
Inulin	-	-
Melezitose	-	-
Starch	-	-
Glycogen	-	-
Xylitol	-	-
β-gentibiose	V	-
D-turanose	-	-
D-tagatose	-	-
L-fucose	-	-
L-arabitol	-	-
2-ketogluconate	-	V
5-ketogluconate	-	-

[*]Data on *B. andropogonis* were based on Viallard *et al.* (1998) and Hu *et al.* (1991)

Chapter 4 Cell Production of Bacterium CW00B006C

4.1. Abstract

This bacterium is easily cultured in submerged conditions. Cell production was significantly affected by the liquid culture media. No lag phase was observed in nutrient glucose broth (NGB), tryptic soy dextrose (TSD), and King's medium B (KB) and about a 2-4 h lag phase was observed in nutrient broth yeast extract (NBY). Exponential growth ended around 22-24 h in all four media tested. Death phase was observed at 42 h in KB and 56 h in NGB and TSD. Growth rate was not significantly different among four media tested, but initial cell production in NBY, NGB, and TSD were similar and significantly greater than that in KB. Estimated generation time (g) was 2.9 h in KB, 2.7 h in NBY, 2.9 h in NGB, and 3.0 in TSD. Medium pH did not affect cell production of *B. andropogonis*. Addition of chickweed extract reduced cell production by 50% when culture time was more than 48 h.

4.2. Introduction

The development of low-cost methods for mass production of a bioherbicide is an important step for the commercialization of a bioherbicide (Stowell et al., 1989; Boyette et al., 1991). Submerged liquid culture spore production is the preferred technique for mass production of biocontrol agents because the technology is readily available and the scale-up process from the research phase to the development phase is relatively easy (Churchill, 1982; Stowell et al., 1989; Jackson, 1997). So far, no information has been reported on submerged liquid culture cell production for *Burkholderia andropogonis*. The objectives of this study were (1) to characterize the growth curve of *B. andropogonis* on different liquid culture media, (2) to determine the effect of medium pH on cell production of *B. andropogonis*, and (3) to evaluate the effect of addition of chickweed extracts on cell production of *B. andropogonis*.

4.3. Materials and Methods

For all experiments, stock cultures of CW00B006C were stored in 15% glycerol at -80 °C. A cryovial of stock culture was warmed to room temperature in a 36°C water bath. A 50 µl aliquot of suspension from the vial was added to 18 x 150 mm glass test tube

containing 3 ml of appropriate broth. Test tubes were incubated on an orbit shaker at 200 rpm for 24 h under ambient laboratory conditions (24 °C ± 3). Contents of tubes of the same medium were combined to produce 'seed inoculum'. Replicate 500 ml flasks of each medium were inoculated with 1 ml of the appropriate 'seed inoculum'. Flasks were placed on an orbit shaker at 200 rpm under ambient laboratory conditions (24 °C ± 3). Viable bacterial cell production was determined using the dilution plate count method. Serial dilutions (10x) were performed in dilution tubes containing 9 ml of sterile 0.01 M phosphate buffer, pH 7, and 100 µl of three appropriate dilutions were spread plated on nutrient glucose agar (NGA). Plates were incubated under ambient laboratory conditions for about 4 days. Colony forming units (CFU) per ml was determined by counting colonies on plates with 10-600 CFU per plate.

4.3.1. Growth curve of the bacterium in different liquid culture media

The growth of the bacterium in four different liquid culture media was assessed over a 44 h period. Liquid culture medium tested included nutrient glucose broth (NGB; 8 g nutrient broth (Difco), 2.5 g glucose, 1 L distilled water), nutrient broth yeast extract (NBY; 8 g nutrient broth (Difco), 2 g yeast extract (Difco), 2 g K_2HPO_4, 0.5 g KH_2PO_4, 2.5 g glucose, 1 L distilled water), King's medium B (KB; 20 g proteose peptone #3 (Difco), 1.5 g. K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, 15 ml glycerol, 1 L distilled water), and tryptic soy dextrose (TSD; 17 g Bacto tryptone (Difco), 3 g Bacto Soytone (Difco), 5 g NaCl, 2.5 g K_2HPO_4, 2.5 g glucose, 1 L distilled water). Two replicate 500 ml flasks of each medium were inoculated with 1 ml of the appropriate 'seed inoculum'. A 0.5 ml sample from each of the two replicate flasks was taken at 0, 2, 4, 6, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 38, 40, 42 and 44 h after inoculation. The 2-0.5 ml samples from each medium were combined and viable bacterial cell concentration was determined using the dilution plate count method.

Since the death phase were not observed within 44 h culture time, an extension of culture time to 64 h was used to culture CW00B006C in NGB and TSD. Two trials were conducted. For each trial, 3 replicate 500 ml flasks of each medium were inoculated with 1 ml of appropriate 'seed inoculum'. A 1 ml sample from each replicate flask was taken every 4 hours from 0 to 44 hrs in trial 1 and from 0 to 48 hours in trial 2, and at 56 and 64 hours for both trials. Each sample was separately analyzed using the dilution plate count

method to determine viable bacterial cell concentration. Data from the two trials of each experiment were pooled because the variances of trials were homogeneous according to Bartlett's test (Gomez & Gomez, 1984).

4.3.2. Effect of medium pH on bacterial cell production

A cryovial of stock culture was warmed to room temperature in a 36°C water bath. A 50 µl aliquot of suspension from the vial was added to each 15 mm glass test tube containing 3 ml nutrient glucose broth (NGB) at a pH of 6.8. The test tubes were incubated on an orbit shaker at 200 rpm for 24 h under ambient laboratory conditions (24 °C ± 3). Contents of tubes were used as 'seed inoculum'. The pH of NGB flasks was adjusted using 1 N HCl or NaOH to achieve pHs from 6 to 8 at increments of 0.2 prior to sterilization. Two replicate flasks of each treatment were inoculated with the 'seed inoculum' and incubated on an orbit shaker at 200 rpm for 24 h under ambient laboratory conditions (24 °C ± 3). Medium pH of each flask after 24 h culture was measured and compared to the original pH. Bacterial cell production was then determined.

4.3.3. Effect of growth medium modification with chickweed extracts.

For all experiments, nutrient glucose broth (NGB) at a pH of 6.8 was used as a control treatment to compare the cell production in chickweed extract medium (CWE). To prepare chickweed extract medium, 3-5 wk old chickweed foliage was harvested from the greenhouse and stored at –20°C until use. Using liquid nitrogen and a pestle and mortar, tissue was crushed to a powder, and combined with an equal amount of distilled water (w/v) to form thick slurry. The slurry was filtered through 2-ply cheesecloth, filtrate was centrifuged at 18500 rcf (Eppendorf 5810R centrifuge) for 15 minutes, supernatant was filter sterilized using a 0.22 µm bottle-top vacuum filter, and resulting sterile CWE medium was stored at 4°C until use. Stock cultures of isolate CW00B006C were stored in 15% glycerol at –80 °C. For each experiment, a cryovial of stock culture was warmed to room temperature in a 36°C water bath. A 50 µl aliquot of suspension from the vial was added to each 18 x 150 mm glass test tube containing 3 ml of appropriate medium. The test tubes of were incubated on an orbit shaker at 200 rpm for 24 h under ambient laboratory conditions (24 °C ± 3). Contents of tubes were used as 'seed inoculum'. The bacterial culture for inoculations was produced using 75 ml of sterile NGB or CWE in

250-ml Erlenmeyer flasks inoculated with 0.5 ml per flask of the appropriate 'seed inoculum'. Unless otherwise indicated, flasks were incubated on an orbit shaker at 200 rpm for 24 h under ambient laboratory conditions (24 °C ± 3). When stated, viable bacterial cell production was determined using the dilution plate count method. Serial dilutions (10x) were performed in dilution tubes containing 9 ml of sterile 0.01 M phosphate buffer, pH 7, and 100 µl of three appropriate dilutions were spread plated on nutrient glucose agar (NGA). Plates were incubated under ambient laboratory conditions for about 4 days. Colony forming units (CFU) per ml was determined by counting colonies on plates with 10-200 CFU per plate.

4.4. Results and Discussions

4.4.1. Growth curve of the bacterium in four different liquid culture media

Isolate CW00B006C grew well on all four media tested (Figure 4.1). No lag phase was observed on NGB, TSD, and KB and about a 2-4 h lag phase was observed on NBY. Exponential growth ended around 22 – 24 h on all four media tested. Simulation of the exponential growth during this period indicates initial cell production on NBY, NGB, and TSD were similar but significantly greater than that on KB (Figure 4.2; Table 4.1). However, growth rate was not significantly different among the four media tested. Generations of 6.6, 6.4, 7.1, and 6.4 from 2 h to 22 h were observed in KB, NBY, NGB, and TSD, respectively. Estimated generation time (g) was 2.9 h in KB, 2.7 h in NBY, 2.9 h in NGB, and 3.0 in TSD. The stationary phase in all four media tested began at 22-24 h and extended to the end of the testing period at 44 h for NBY, NGB and TSD. Death phase was observed only on KB at 42 h.

Since the death phase were not observed within 44 h culture time, an extension of culture time to 64 h was used to culture CW00B006C in NGB and TSD. Results demonstrated that the growth curve of CW00B006C in both media follows a similar pattern. The death phase began at 56 h (Figure 4.3).

4.4.2. Effect of medium pH on bacterial cell production

Medium pH did not significantly affect the growth curve of isolate CW00B006C, or the bacterial cell production. A slight decrease in cell production was observed with an increase in pH. The highest number of bacterial cells, 9.2 x 10^{10} CFU/ml, was produced

at a pH of 6 while the lowest number of bacterial cells, 2.9 x 10^{10} CFU/ml, was produced at a pH of 8.

4.4.3. Effect of growth medium modification with chickweed extracts

Since the addition of chickweed extract in culture medium enhanced the efficacy of *B. andropogonis* for the control of chickweed, the bacterial cell production was assessed in the culture medium modified with the chickweed extract (Figure 4.4). Cell production in CWE was similar to NGB within 24 h. However, when culture time extended to 48 h or 72 h, cell production in CWE was half of that in NGB. Further study is required to balance the cell production and weed control efficacy.

4.5. Literature Cited

1. Boyette, C.D., Quimby, P.C. Jr., Connick, W.J., Daigle, D.J. and Fulgham, F.E. 1991. Progress in the production, formulation, and application of mycoherbicide. Pages 209-222 *in* TeBeest, D.O. (ed) Microbial Control of Weeds. Chapman & Hall, New York.
2. Churchill, B.W. 1982. Mass production of microorganisms for biological control. Pages 139-156 *in* Charudattan, R. and Walker, H.L. (eds) Biological Control of Weeds with Plant Pathogens. John Wiley & Sons, New York.
3. Gomez, K.A. and Gomez, A.A. 1994. Statistical Procedures for Agricultural Research. 2nd Edition. John Wiley & Sons, Inc. New York.
4. Jackson, M.A. 1997. Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents. J. Ind. Microbiol. Biotech. 19:180-187.
5. SAS Institute Inc. 1987. SAS/STAT Guide for personal computers. Version 6. SAS Institute Inc., Cary, NC.
6. Stowell, L.J., Nette, K., Heath, B. and Shutter, R. 1989. Fermentation alternatives for commercial production of a mycoherbicide. Pages 219-227 *in* Demain, A.L., Somkuti, G.A., Hunter-Cevera, J.C. and Rossmoore, H.W. (eds) Novel Microbial Products for Medicine and Agriculture. Society for Industrial Microbiology.

Table 4.1 Comparison of exponential growth parameters of CW00B006C among four liquid culture media*

Medium	Interception**	Slope**	Generation time g
King's medium B	6.968 b	0.105 a	2.87
Nutrient broth yeast extract	7.437 a	0.110 a	2.74
Nutrient glucose broth	7.407 a	0.104 a	2.89
Tryptic soy dextrose	7.372 a	0.099 a	3.04

* Cell numbers from 2 h to 22 h were used to do the regression between cell numbers and culture times.

** Values in each column sharing the same letter are not significantly different according to the t-test.

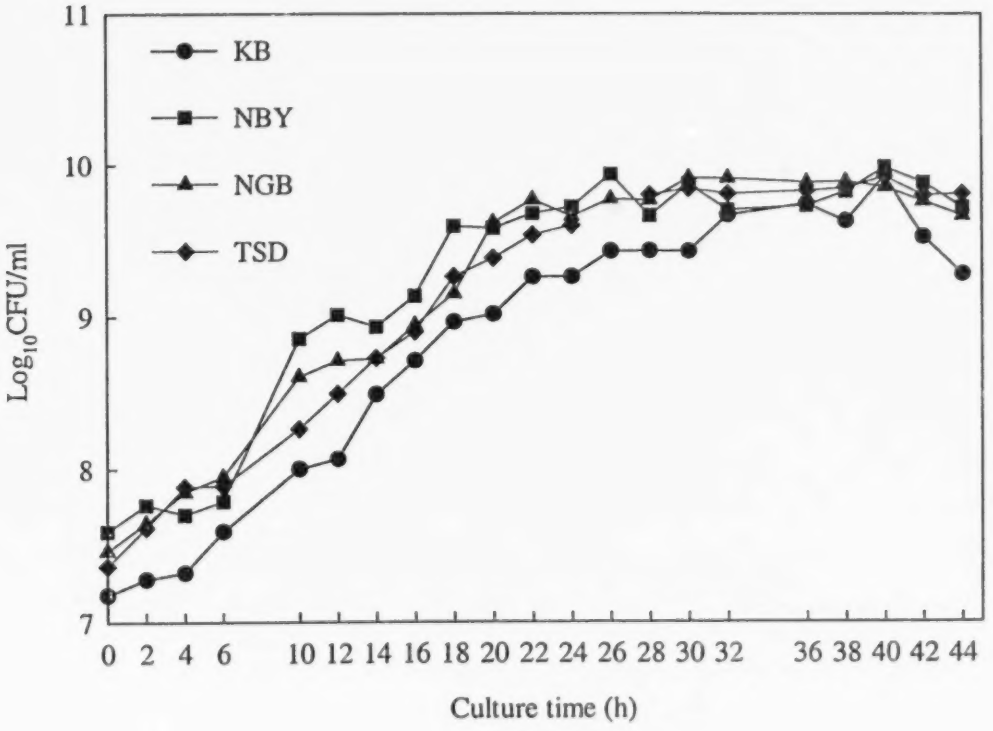

Figure 4.1 CW00B006C bacterial cell growth curve in four different liquid culture media over 44 h period. KB - King's medium B, NBY - nutrient broth yeast extract, NGB - nutrient glucose broth, TSD - tryptic soy dextrose.

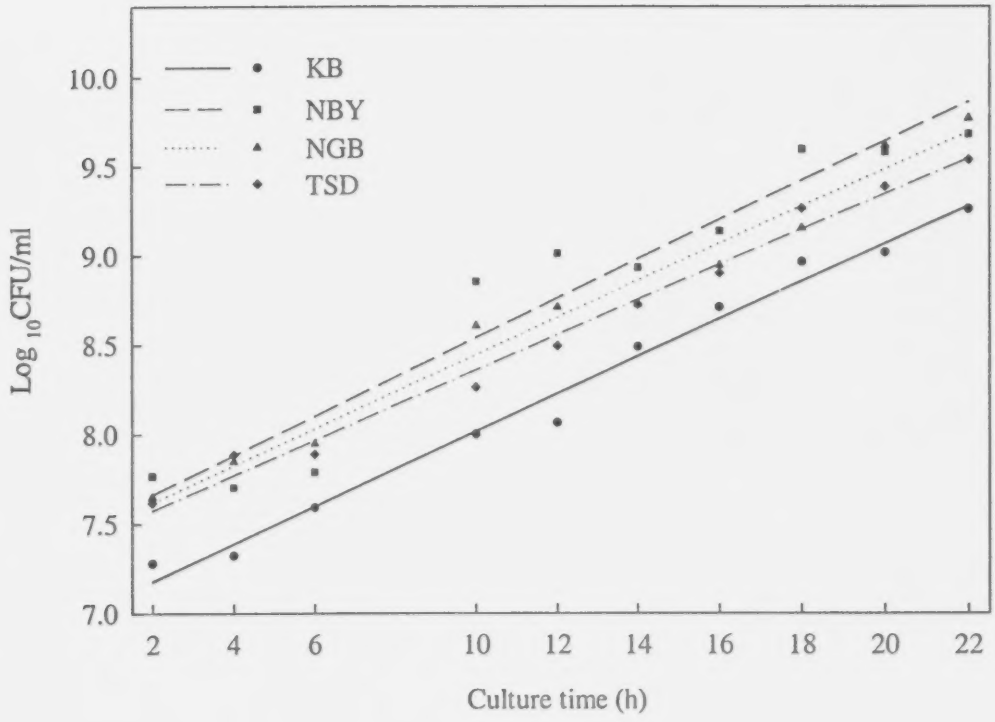

Figure 4.2 Exponential growth of CW00B006C bacterial cells in four different liquid culture media from 2 to 22 44 h. KB - King's medium B, NBY - nutrient broth yeast extract, NGB - nutrient glucose broth, TSD - tryptic soy dextrose. The best regression equation between cell numbers (N) and time (T) are: (a) For KB, $N = 6.968 + 0.105T$, $r^2 = 0.9868$, (b) For NBY, $N = 7.437 + 0.110T$, $r^2 = 0.9317$, (c) For NGB, $N = 7.407 + 0.104T$, $r^2 = 0.9762$, and (d) For TSD, $N = 7.372 + 0.099T$, $r^2 = 0.9873$.

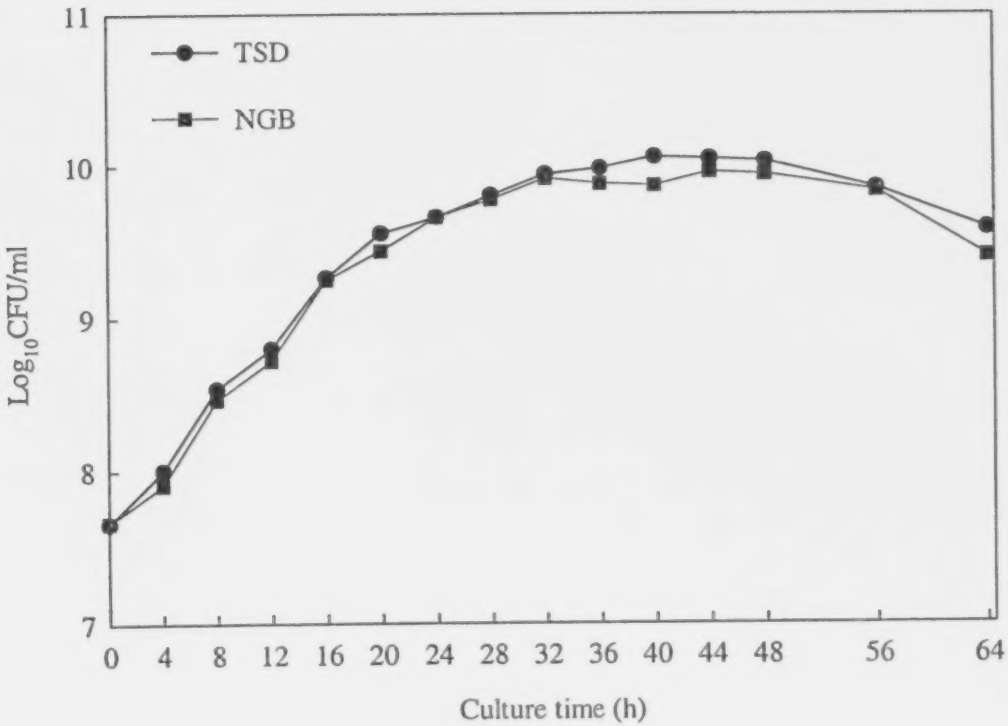

Figure 4.3 CW00B006C bacterial cell growth curve in two different liquid culture media over 64 h period. NGB - nutrient glucose broth, TSD - tryptic soy dextrose. Data from the two trials of each experiment were pooled because the variances of trials were homogeneous according to Bartlett's test (Gomez & Gomez, 1984). Data points represent means of six replications.

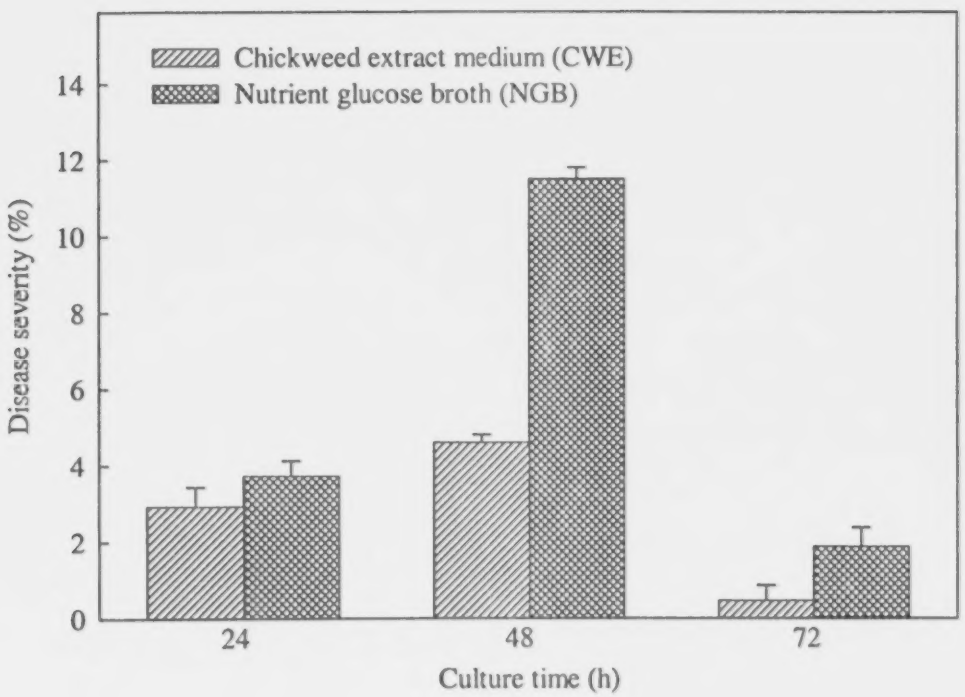

Figure 4.4 Effect of chickweed extract on CW00B006C bacterial cell production.

Chapter 5 Host Range

5.1. Abstract

Thirty-six plant species in 30 genera and 8 families, selected by using the centrifugal phylogenetic method, were screened against the bacterial strain *Burkholderia andropogonis* (CW00B006C), a bioherbicide candidate collected in Alberta, Canada from diseased chickweed. Trials were performed under greenhouse conditions. Plants were inoculated with a bacterial suspension at 10^9-10^{10} CFU/ml plus a surfactant, Silwet L-77®. This bacterial strain of *B. andropogonis* caused disease of host plants distributed in the Caryophyllaceae, Poaceae, and Fabaceae families. The number of species in the Caryophyllaceae family that this bacterial strain was able to infect is relatively wide including chickweed and six other weeds common in western Canada, expanding *B. andropogonis* weed control spectrum as a bioherbicide. In the Poaceae family, corn is susceptible to this strain of bacterium. In the Fabaceae family, hosts of our *B. andropogonis* strain were restricted to Tribe Vicieae containing common vetch and chickpea. Our strain of *B. andropogonis* is not pathogenic to the majority of major economically important crops cultivated in western Canada. On the basis of host specificity, the use of *B. andropogonis* as a bioherbicide for control of common chickweed will not cause major concerns to crops and native flora in Canada. However, bioherbicide formulations containing *B. andropogonis* should not be applied to susceptible plants such as corn, chickpea, carnation, and tulip.

5.2. Introduction

Host specificity of a biocontrol agent is an important factor to consider in a biological weed control program (Watson, 1985). Before using a biological control agent in the field, its host specificity to non-target economic and wild plants must be characterized (Wapshere, 1974).

Various plant species were reported as hosts of *B. andropogonis*. However, cross inoculation studies demonstrated variations in the host reactions of isolates from a particular host genus or species, indicating a degree of host specialization associated with *B. andropogonis* strains (Moffett et al., 1986). According to Moffett et al. (1986), common hosts of *B. andropogonis* include sorghum (*Sorghum bicolor*) and corn (*Zea mays*) of the family Poaceae, common vetch (*Vicia sativa*) of the family Fabaceae, and

carnation (*Dianthus caryophyllus*) and baby's breath (*Gypsophila paniculata*) of the family Caryophyllaceae. Other possible hosts, depending upon the strain, included sudan grass (*Sorghum sudanens*) of the family Poaceae, chick pea (*Cicer arietinum*), velvet bean (*Mucuna deeringiana*), white clover (*Trifolium repens*), and red clover (*Trifolium pratense*) of the family Fabaceae, blueberry (*Vaccinium* sp.) of the family Rubiaceae, and tulip (*Tulip sylvestris*) of the family Liliaceae (Burkholder, 1957; Allen et al., 1970; Goto and Starr, 1971; Hayward, 1972; Nishiyama et al., 1979; Caruso, 1984).

Burkholderia andropogonis has never been reported as a crop pathogen in Canada (Howard et al., 1994; Martens et al., 1994). The host range of the Canadian strain of *B. andropogonis* is unknown. Thus, the objectives of this study were to determine the host range of this strain of *B. andropogonis* based on the phylogenetic method and to estimate the potential risks involved in its use as a biological control agent of common chickweed in western Canada.

5.3. The organisms

5.3.1. The target weeds: common chickweed

Common chickweed is native to Europe (Holm et al., 1977; Turkinton et al., 1980). It is believed to be one of the 12 most successful colonizing species among the noncultivated plants and is reported to be a weed in more than 20 crops in 50 countries (Holm et al., 1977). It is an annual or winter annual, spreading by seed and the stems rooting at the nodes. Stems are slender, round, branching, usually prostrate and spreading with lines of fine hairs and swollen nodes at the leaf base. Leaves are in pairs on opposite sides of the stem at each node, the lower and middle leaves are stalked and the stalk often has a fringe of hairs along each edge, upper leaves are stalkless and are oval with pointed tips. Flowers are located in the axils of the leaves, about 6 mm across with five sepals and five white petals, flowering throughout the growing season and maturing seed rapidly after flowering. Seeds are reddish brown to tan colored, about 1 mm across, and covered with regular rows of small bumps. Common chickweed grows best on moist, heavy, nitrogen rich soils at optimum, constant temperature of 12-20 °C (Turkinton et al., 1980). In Canada, common chickweed can germinate throughout the growing season but has two main flushes: one is early spring while the other is in late fall.

Common chickweed belongs to the genus *Stellaria*, subfamily Alisnoideae, family Caryophyllaceae, order Caryophyllales (Cronquist 1981). The family Caryophyllaceae consists of three subfamilies including Alsinoideae, Paronychioideae, and Silenoideae containing 13, 12, and 14 genera, respectively (Cronquist, 1981; Jalas and Suominen, 1987). The most familiar species in the subfamily Alsinoideae include the weeds such as common chickweed, mouse-eared chickweed (*Cerastium vulgatum* L.), and Knawel weed (*Scleranthus annus* L.). Subfamily Paronychioideae contains the weed Corn spurry (*Spergula arvensis* L.). Subfamily Silenoideae contains a number of garden ornamentals, such as carnation (*Dianthus caryophyllus* L.) and baby's breath (*Gypsophila sp.*), as well as weeds such as *Gypsophila paniculata* L., white cockle (*Lychynis alba* Mill.), cow cockle (*Saponaria vaccaria* L.), bladder Campion (*Silene cucubalis* Wibel), and night-flowering catchfly (*Silene noctiflora* L.).

The Order Caryophyllales has 11 families with major economic importance as both garden ornamentals and notable weeds (Bateman, 1985; Jalas and Suominen, 1987a; 1987b). Considering the phenogenetic relationship to common chickweed, important plants in families other than Cayophyllaceae family may include weeds, such as *Chenopodium* spp., *Amaranthus* spp., common purslane (*Portulaca oleracea* L.), and Russian thistle (*Salsola kali* L. var. *tenuifolia* Tausch), as well as vegetables, such as beet (*Beta vulgaris* L.) and spinach (*Spinacea oleracea* L.). Plants native to Canada in all families of the order Caryophyllales grow exclusively in non-cultivated areas (Scoggan, 1978). Few are common in agricultural fields, home gardens, or golf courses (Table 5.3).

5.3.2. The biocontrol agent: *Burkholderia Andropogonis*

Burkholderia andropogonis (Smith 1911) Gillis *et al.* 1995, comb. nov. belongs to the family Burkholderiaceae, order Burkholderiales, class Betaproteobacteria, Phylum Proteobacteria (Boone et al., 2001). Several synonyms exist as *Pseudomonas andropogonis* (Smith 1911) Stapp 1928, *Bacterium andropogon* (*sic*) Smith 1911, *Aplanobacter stizolobii* Wolf 1920, *Pseudomonas stizolobii* (Wolf 1920) Stapp 1935 (Gillis, 1995; Viallard et al., 1998). *B. andropogonis* appears in Group A (isolated from diseased plants and nonfluorescent), Section V of *Pseudomonas* in Bergey's Manual (Palleroni, 1984). *B. andropogonis* has been reported as pathogenic to sorghum, corn, clover, velvet bean, and carnation. Eight other bacterial species in the same group (Group

A) were reported as pathogenic to the bird's-nest fern (*Asplenium nidus*), pawpaw, cultivated mushroom, almond tree (*Prunus dulcis*), oats (*Avena sativa*), foxtail (*Chaetochloa lutescens*), *Ciccus japonica*, *Cattleya* sp., *Phalaenopsis* sp., tomato, rice, and sugarcane.

5.4. Methods and Materials

5.4.1. Inoculum preparation

Stock cultures of *B. andropogonis* were stored in 15% glycerol at -80 °C. For each experiment, a cryovial of stock culture was warmed to room temperature in a 36°C water bath. A 50 µl aliquot of suspension from the vial was added to each 18 x 150 mm glass test tube containing 3 ml nutrient glucose broth (NGB) at a pH of 6.8. The test tubes were incubated on an orbit shaker at 200 rpm for 24 h under ambient laboratory conditions (24 °C ± 3). Contents of tubes were used as 'seed inoculum'. Unless otherwise stated, the bacterial culture for inoculations was produced by placing 75 ml of NGB (pH 6.8) in each 250-ml Erlenmeyer flasks, autoclaving, cooling, inoculating with 1 ml per flask of the 'seed inoculum', and incubating flasks on an orbit shaker at 200 rpm for 24 h under ambient laboratory conditions (24 °C ± 3). When stated, viable bacterial cell production was determined using the dilution plate count method. Serial dilutions (10x) were performed in dilution tubes containing 9 ml of sterile 0.01 M phosphate buffer, pH 7, and 100 µl of three appropriate dilutions were spread plated on nutrient glucose agar (NGA). Plates were incubated under ambient laboratory conditions for about 4 days. Colony forming units (CFU) per ml was determined by counting colonies on plates with 10-200 CFU per plate.

5.4.2. Plant production

Using the modified centrifugal phylogenetic and varietal strategy (Wapshere, 1974), 36 plant species in 30 genera and 8 families were selected for the host range trial (Table 5.1). Test plants were grown from seed or propagated vegetatively depending on the species being tested and availability of material. Plants were planted in 10-cm diameter peat pots containing steam-pasteurized soil mix. The soil mix consisted of 140 L soil (loam, pH 6.7, 42% sand, 40% silt, and 18% clay), 107 L sand, 160 L Sunshine Growing Mix (SunGro Horticulture, Bellevue, Washington), 62 L Fibrous Blond Shagnum Peat Moss (Premier Pro Moss, Riviere-du-Loup, Quebec), 113 L vermiculite (Therm-O-Rock, Chandler, Arizona),

230 g dolomite lime, and 150 g Super Phosphate (0-45-0) per batch of soil. Seeded pots were placed in a greenhouse with 23/20 ± 4°C day/night temperature, a 16 h photoperiod, an average light intensity of 300 $\mu Em^{-2}s^{-1}$, and an average relative humidity of 45-50%. After germination, seedlings were thinned to five plants per pot.

5.4.3. Treatments

For each species, two sets of test plants (each set containing 15 plants) were prepared. One set was inoculated with *B. andropogonis* and the other set served as uninoculated controls. Seedlings of selected plant species at the 2- to 3-leaf stage were arbitrarily selected. For the inoculated treatment, seedlings were sprayed with the bacterial culture suspension at 10^9-10^{10} CFU/ml bacterial cells plus 0.15% Silwet L-77®, using an H-set airbrush (Paasche Airbrush Company, Harwood Heights, IL) at a pressure of 100 kPa. The application volume of the culture suspension was 5 ml/pot. For uninoculated control, seedlings were sprayed with 0.15% Silwet L-77®. Immediately after spraying, pots were returned to the greenhouse and placed in a randomized block design for the remainder of the experiment.

5.4.4. Measurements

Disease severity was visually assessed daily until the 14th day after inoculation by using the 0-11 rating scale by Horsfall and Barrett (1945). Chickweed seedlings at the 4-6 leaf growth stage were also inoculated with the same inoculum and served as a control. The experiment was conducted as a factorial experiment with species tested and inoculation levels (0 and 10^9-10^{10} CFU ml^{-1}) as factors. Each treatment was replicated three times.

5.5 Results and Discussions

Burkholderia andropogonis has never been reported as a pathogenic bacterium causing crop diseases in Canada (Howard et al., 1994; Martens et al., 1994). To our knowledge, this is first description of *B. andropogonis* as a plant pathogen in Canada (Howard et al., 1994; Martens et a' , 1994). Host specificity test for 36 plant species in 30 genera and 8 families demonstrated that our strain of *B. andropogonis* is not pathogenic to the majority of major economically important crops cultivated in western Canada. Table 3 shows the plant species with disease ratings greater than 0.

Findings in the study demonstrated that hosts of our *B. andropogonis* strain isolated from common chickweed were distributed in the families Caryophyllaceae, Poaceae, and Fabaceae. No infection on clover confirmed that our bacterial isolate possesses host specialization, supporting the conclusions reported by Moffett et al. (1996). Hosts in the Caryophyllaceae family of our *B. andropogonis* strain are relatively wide (Table 5.3). All plant species in the Silenoideae subfamily was diseased by the inoculation of *B. andropogonis*. In the Alsinoideae subfamily, all seedlings of common chickweed serving as positive controls were severely diseased. However, the other plant species in the same subfamily, corn spurry (*Spergula arvensis*), was not affected by the inoculation of *B. andropogonis*. In the Paronychioideae subfamily, the selected plant species, Knawel weed (*Scleranthus annus*) was not affected by the inoculation of *B. andropogonis*. Results demonstrated that this bacterium also causes disease at various levels on six other weed species common to western Canada in this family, expanding *B. andropogonis'* weed control spectrum as a bioherbicide. On the other hand, potential risks to carnation (*Dianthus caryophyllus*) and other ornamental plants in this family should be further determined.

In the Poaceae family, three plant species including corn (*Zea Mays*), sorghum (*Sorghum bicolor*), and sudan grass (*Sorghum sudanens* (Piper) Stapf.) were reported as hosts of *B. andropogonis* in the United States (Smith & Hedge, 1905; Ullstrup, 1960). Our results demonstrated that although a water-soaked lesion was observed on two of nine corn seedlings treated with *B. andropogonis*, disease ratings on bacterial treated corn seedlings were similar to that on untreated corn seedlings two weeks after inoculation. Three weeks after inoculation, no visible difference was observed between treated and untreated corn seedlings. Ullstrup (1960) reported that *B. andropogonis* caused bacterial stripe of a few very susceptible inbred corn lines and their progenies but not on commercial field corn in the United States. Our results confirmed that our selected commercial corn variety was not significantly susceptible to *B. andropogonis* to allow development of the typical bacterial stripe symptoms. Extensive tests on major commercial corn varieties currently cultivated in North America are necessary to further define the risks associated with the use of this bacterium for control of common chickweed. Similar tests on sorghum and sudan grass are also warranted.

In the Fabaceae family, hosts of our *B. andropogonis* strain were restricted to Tribe Vicieae among 50 tribes (Hutchinson, 1967). Susceptible species in this Tribe include common vetch and chickpea. However, another economically important crop in this Tribe, lentil (*Lens culinaris*), was unaffected by this bacterium. Other major crops in this family were also unaffected by this bacterium.

Although *B. andropogonis* has a relatively wide plant host range, wounds plus infiltration are usually pre-requirements for the infections (Moffett et al., 1986). The absence of these prerequisites in the natural Canadian environment may explain why *B. andropogonis* has never been reported as a plant pathogen. Bioherbicide preparation of *B. andropogonis* contains a special surfactant, Silwet L-77®, that allows infiltration of bacterial cells into plant tissues without the presence of wounds (Zidack et al., 1992). Therefore, application of *B. andropogonis* as a bioherbicide will cause disease only on directly sprayed target weed species but not on other susceptible plant species under natural conditions. Since plants native to Canada in all families of the order Caryophyllales grow exclusively in non-cultivated areas (Scoggan, 1978) and few are common in agricultural field, home gardens, or golf courses (Table 5.3). Therefore, application of *B. andropogonis* as a bioherbicide will not cause disease on native plant species under natural conditions.

In conclusion, the use of *B. andropogonis* as a bioherbicide for control of common chickweed will not cause major concerns to crops and native flora in Canada. However, bioherbicide formulations containing *B. andropogonis* should not be applied to susceptible plants such as corn, chickpea, carnation, and tulip.

5.6. Literature Cited

1. Allen, R.N., A.C. Hayward, W.J. Halliday and J. Fulcher. 1970. Bacterial blight of Vicia sativa: aetiology of disease and identification of the pathogen. Australian Journal of Biological Science 23:597-606.
2. Bateman, G. 1985. Flowering Plants of the World. Croom Helm Publishers Ltd, Beckenham, Kent, UK. pp.63-76.
3. Burkholder, W.H. 1957. A bacterial disease of clover and velvet beans. Phytopathology 47:48-50.

4. Caruso, F.L. 1984. Bacterial blight of chickpea incited by *Pseudomonas andropogonis*. Plant Disease 68:910-913.
5. Coenye, T., Laevens, S., Gillis, M. and Vandamme, P. 2001. Genotypic and chemotaxonomic evidence for the reclassification of *Pseudomonas woodsii* (Smith 1911) Stevens 1925 as *Burkholderia andropogonis* (Smith 1911) Gillis *et al.* 1995. *Int. J. Syst. Evol. Microbiol.*, **51**, 183-185.
6. Cronquist, A. 1981. An Integrated System of Classification of Flowering Plants. Columbia University Press, New York. Pp. 231-276.
7. Gillis, M., Van, T.V., Bardin, R., Goor, M., Hebbar, P., Willems, A., Segers, P., Kersters, K., Heulin, T., and Fernandez, M.P. 1995. Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N2-fixing isolates from rice in Vietnam. *Int. J. Syst. Bacteriol.*, **45**, 274-289.
8. Goto, M. and M.P. Starr. 1971. A comparative study of *Pseudomonas andropogonis*, *P. stizolobii* and *P. alboprecipitans*. Annals of the phytopathological Society of Japan 37:233-241.
9. Hayward, A.C. 1972. A bacterial disease of clover in Hawaii. Plant Disease Reporter 56:446-450.
10. Horsfall, J.G. and R.W. Barrett .1945. An improved grading system for measuring plant diseases. Phytopathol. 35:655.
11. Holm, L.G., Plucknett, D.L., Pancho, J.V. and Herberger, J.P. 1977. The world's worst weeds: distribution and biology. The University Press of Hawaii, Honolulu, Hawaii.
12. Howard, R.J., J.A. Garland and W.L. Seaman. 1994. Diseases and pests of vegetable crops in Canada: an illustrated compendium. The Canadian Phytopathological Society and Entomological Society of Canada. Ottawa, Ontario. Pp.554.
13. Hutchinson, J. 1964. The genera of flowering plants (Angiospermae), based principally on the Genera plantarum of G. Bentham and J.D. Hooker / by J. Hutchinson. Oxford at the Clarendon Press. Pp452-454.
14. Jalas J. and Suominen J. 1987a. Atlas Florae Europaeae: Distribution of vascular plants in Europe III. 6. Caryophyllaceae (Alsinoideae and Paronychioideae). Cambridge University Press, Cambridge Cb2 1RP. Pp. 1-168.

15. Jalas J. and Suominen J. 1987b. Atlas Florae Europaeae: Distribution of vascular plants in Europe III. 6. Caryophyllaceae (Alsinoideae and Paronychioideae) and 7. Caryophyllaceae (Silenoideae). Cambridge University Press, Cambridge Cb2 1RP. Pp. 1-220.

16. Martens, J.W., W.L. Seaman, and T.G. Atkinson. 1994. Diseases of Field Crops in Canada: an Illustrated Compendium. The Canadian Phytopathological Society. Ottawa, Ontario. Pp.160.

17. Moffett, M.L., A.C. Hayward and P.C. Fahy. 1986. Five new hosts of Pseudomonas andropogonis occurring in eastern Australia: host range and characterization of isolates. Plant Pathology 35:34-43.

18. Moss, E.H. 1983. Flora of Alberta: A manual of flowering plants, Conifers, Ferns and Fern Allies found growing without Cultivation in the Province of Alberta, Canada. Pp253-260.

19. Nishiyama, K., T. Kusaba, K. Ohta, K. Nahata and K. Ezuka. 1979. Bacterial black rot of tulip caused by Pseudomonas andropogonis. Annals of the Phytopathological Society of Japan 45:668-674.

20. Palleroni, N.J. 1984. Genus I. Pseudomonas Migula 1894. In Bergey's Mannual of Systematic Bacteriology, vol. 1., pp. 141-199. Edited by N.R. Krieg & J.G. Holt. Baltimore: Williams and Wilkins.

21. Scoggan, H.J. 1978. Caryophyllaceae in The Flora of Canada Part 3 – Dicotyledoneae (Saururaceae to Violaceae). National Museums of Canada, Ottawa. Pp. 673-710.

22. Turkington, R., Kenkel, N.C., and Krankko, G.D. 1980. The biology of Canadian weeds. 42. *Stellaria media* (L.) Vill. *Can. J. Plant Sci.* 60:981-992.

23. Viallard, V., I. Poirier, B. Cournoyer, J. Haurat, S. Wiebkin, K. Ophel-Keller and J. Balandreau. 1998. *Burkholderia graminis* sp. nov., a rhizospheric *Burkholderia* species, and reassessment of [*Pseudomonas*] *phenazinium*, [*Pseudomonas*] *pyrrocinia* and [*Pseudomonas*] *glathei* as *Burkholderia*. Int. J. Syst. Bacteriol. 48: 549-563.

24. Wapshere, A.J. 1974. A strategy for evaluating the safety of organisms for biological weed control. Ann. Appl. Biol. 77:201-211.

25. Watson, A.K. 1985. Host specificity of plant pathogens in biological weed control. *In* Proceedings of the 6th International Symposium on Biological Control of Weeds,

August 19-25, 1984, Vancouver, B.C. Edited by E.S. Delfosse. Agriculture Canada, Ottawa, Ont. Pp.577-586.

26. Ullstrup, A.J. 1960. Bacterial strip of corn. Phytopathology 50:906-910.

Zidack, N.K., Backman, P.A., and Shaw, J.J. 1992. Promotion of bacterial infection of leaves by an organosilicone surfactant: implications for biological weed control. Biological Control 2:111-117.

Table 5.1 Test plant species used for host-specificity screening of *Burkholderia andropogonis* against common chickweed (*Stellaria media*) based on the modified centrifugal phylogenetic and varietal strategy

Caryophyllaceae

Subfamily Alsinoideae

1. *Stellaria media* (L.) Vill. (Chickweed)
2. *Scleranthus annus* L. (Knawel weed)

Subfamily Paronychioideae

3. *Spergula arvensis* L. (Corn spurry)

Subfamily Silenoideae

4. *Dianthus caryophyllus* L. (Carnation)
5. *Gypsophila paniculata* L. (Baby's-Breath)
6. *Lychynis alba* Mill. (White cockle)
7. *Saponaria vaccaria* L. (Cow cockle)
8. *Silene cucubalis* Wibel (Bladder Campion)
9. *Silene noctiflora* L. (Night-flowering catchfly)

Poaceae

Subfamily Festucoideae

Tribe Hordeae

10. *Triticum aestivum* L. (Wheat, cv. Katepwa)
11. *Hordeum vulgare* L. (Barley, cv. Bridge)
12. *Lolium perenne* (perennial ryegrass, cv. Fiesta II and Low Grow)

Tribe Agrostideae

17. *Agrostis palustris* (creeping bentgrass, cv. Penncross and Cata)

Subfamily Panicoideae

Tribe Aveneae

13. *Avena sativa* L. (Oats, cv. Unknown)

Tribe Festuceae

14. *Festuca arundinacea* Schreb. (Tall fescue, cv. Mustang II and Crossfire)
15. *Festuca rubra* (Creeping red fescue, cv. Boreal and Jasper)
16. *Poa pratensis* (Kentucky bluegrass, cv. Limousine and Merit)

Fabaceae

Tribe Vicieae

21. *Cicer arietinum* L. (Desi chick pea cv. Myles)
22. *Cicer arietinum* L. (Kabuli chickpea cv. Dwelley)
23. *Lens culinaris* Medic. (Lentil, cv. Laird)
24. *Vicia sativa* L. (Common vetch)

Tribe Phasepleae

25. *Pisum sativum* L. (Pea, cv. Radley)

Liliaceae

31. *Tulip sylvestris* L. (Tulip)

Rubiaceae

Tribe Andropogoneae

18. *Sorghum bicolor* L. (Sorghum, cv.)
19. *Sorghum sudanens* (Piper) Stapf. (Sudan grass)

Tribe Maydeae

20. *Zea mays* L. (Corn, cv. Early Golden Bantum)

Tribe Trifolieae

26. *Medicago sativa* L. (Alfalfa, cv. Algonquin)
27. *Trifolium pratense* L. (Red clover)
28. *Trifolium repens* L. (White clover)
29. *Trifolium hybridum* L. (Alsike clover)
30. *Melilotus officinalis* (L.) Pall. (Sweet clover cv. Yukon)

32. *Vaccinium* sp. (Blueberry, cv.)

Linaceae

33. *Linum usitatissimum* L. (Flax, cv. Norlin)

Asteraceae

34. *Helianthus annuus* L. (Sunflower cv. S6140)

35. *Carthamus tinctorius* L. (Safflower cv. unknown)

Brassicaceae

36. *Brassica napus* L. (Argentine Canola)
    cv. Invigor 2153 (Liberty Link)
    cv. Quest (Roundup Ready)
    cv. 45A71 (Pursuit Smart)
    cv. Quantum (Conventional)
    cv. Impulse (Conventional)

37. *Brassica rapa* L. (Polish Canola)
    cv. Hysyn 111
    cv. Reward

Table 5.2 Disease severity results of host specificity screening for *Burkholderia andropogonis*

Test plant species[a]	Disease grade[b]
Chickweed (*Stellaria media*)	5.0
Carnation (*Dianthus caryophyllus*)	3.0
White cockle (*Lychynis alba*)	2.0
Cow cockle (*Saponaria vaccaria*)	4.3
Baby's- Breath (*Gypsophila paniculata*)	2.3
Bladder Campion (*Silene cucubalis*)	1.7
Night-flowering catchfly (*Silene noctiflora*)	3.3
Sorghum (*Sorghum bicolor*)	NA
Chick pea (*Cicer arietinum*)	3.0
Common vetch (*Vicia sativa*)	4.3

[a] All other species remained uninfected, severity ratings were 0.

[b] Seedlings of plants at the 2- to 3-leaf stage were inoculated with the bacterial culture suspension at 10^9-10^{10} CFU/ml bacterial cells plus 0.15% Silwet L-77®. The application volume of the culture suspension was 5 ml/pot. Chickweed seedlings at the 4- to 6-leave stage were also inoculated with the same inoculum and served as a control. Disease severity was visually assessed daily until the 14th day after inoculation by using the 0-11 rating scale by Horsfall and Barrett (1945).

Table 5.3 Worldwide distribution, economic importance, and Canadian native flora and their habitats in the Order Caryophyllales[a]

Family	Worldwide Distribution	Economic uses	Canadian native flora and their habitats
Cactaceae	Semi-desert regions of North, central and South America	Some garden and house ornamentals with local uses for fruits	Four species in two genera In dry sands, rocks, hillsides. None in cultivated lands, home gardens, golf courses
Aizoaceae	Pantropical, but centered in South Africa	Many greenhouse and garden ornamentals and ornamental curiosity	None
Phytolacceae	Tropical and subtropical, America and West Indies	Many medicinal uses, yield red dyes and are used as ornamentals and potherbs	One species In damp woods. Not in cultivated lands, home gardens, golf courses
Achatocarpaceae	Tropical and subtropical, America and West Indies	Woods and shrubs	None
Nyctaginaceae	Pantropical	*Bougainvillea* and *Mirabilis* cultivated as ornamentals; Pisonia used as vegetables	Four species in two genera In coastal sands, sandy soil, dry plains, and foothills. None in cultivated lands, home gardens, golf courses
Didieraceae	Dry parts of Madagascar	Rarely cultivated	None
Basellaceae	Tropical America	Limited use as vegetables and ornamentals	None
Portulacaeae	Cosmopolitan, but centered in South Africa and America	Several ornamentals and a potherb	22 species in five genera In gravelly to heavy soils, woods, tundra, slopes, rocks, banks, lowlands. None in cultivated lands, home gardens, golf courses
Chenopodiaceae	Temperate and subtropical, particular in saline habitats	Sugar beet, beetroot, leaf beets, and spinach	22 species in five genera In dry plains, alkaline flats, coastal sands, salt marshes, waste places, shores. None in cultivated lands, home gardens, golf courses

Amaranthaceae	Cosmopolitan, with tropical members centered in Africa and America	Widely cultivated as garden ornamentals and a few used as pot herbs and vegetables	Four species in the genus Amaranthus In alkaline moist flats, lakeshores, waste places. None in cultivated lands, home gardens, golf courses
Caryophyllaceae	Temperate regions, centered in the Mediterranean area	Many popular garden ornamentals, notably pinks and carnations; others are widespread weeds	62 species in 9 general In moist meadows, montane forests, streambanks, hillsides, tundra, rocky places, damp thickets, brackish or saline shores, coastal muds and sands, salt marshes, sandy and gravelly places, barrens, cliffs, ravines, ledges, woodlands, river bars. None in cultivated lands, home gardens, golf courses

[a] The phylogenetic relationship and other data was based on Cronqiust (1981) and Bateman (1985)

Chapter 6 Efficacy

6.1. Abstract

Efficacy of chickweed control with *Burkholderia andropogonis* (bacterial isolate CW00B006C) was assessed under both greenhouse and field conditions. Various factors impacted herbicidal performance of this bacterium against chickweed. Under greenhouse conditions, increasing bacterial cell concentration, repeat application, addition of surfactant Silwet L-77® at 0.1%-0.2%, or application of bacterial inoculum to chickweed seedlings at an older growth stage significantly increased disease severity on chickweed seedlings. However, spray pH did not affect the bacterial performance as a bioherbicide against chickweed. Under field conditions, about 65%-80% disease severity was constantly observed with application of *B. andropogonis* at 10^9-10^{10} CFU/ml plus 0.15% Silwet 77®. A maximum percent dry weight reduction of 79.9% was obtained. Moreover, *B. andropogonis* caused equivalent disease severity on Group 2 herbicide resistant chickweed seedlings, providing a new approach for mitigating herbicide resistance development in chickweed.

6.2. Introduction

After discovery, the key considerations in the decision to develop bioherbicides are efficacy and safety (Watson and Wymore, 1990). Weed control of the candidate agent should be assessed by the speed, amount, and ease of control. However, efficacy should not be determined by weed mortality alone. It has been reported that crop yield or economic threshold may be the most suitable index of bioherbicides because the non-complete eradication of weed populations can cause the significant increases in crop yields. The objective of this study is to quantify the effectiveness of *B. andropognis* for the control of chickweed.

6.3. Materials and Methods

6.3.1. Efficacy under greenhouse conditions

6.3.1.1 Inoculum preparation.

For all experiments, nutrient glucose broth (NGB) at a pH of 6.8 and/or chickweed extract medium (CWE) was used. To prepare chickweed extract medium, 3-5 wk old

chickweed foliage was harvested from the greenhouse and stored at -20°C until use. Using liquid nitrogen and a pestle and mortar, tissue was crushed to a powder, and combined with an equal amount of distilled water (w/v) to form thick slurry. The slurry was filtered through 2-ply cheesecloth, filtrate was centrifuged at 18500 rcf (Eppendorf 5810R centrifuge) for 15 minutes, supernatant was filter sterilized using a 0.22 µm bottle-top vacuum filter, and resulting sterile CWE medium was stored at 4°C until use. Stock cultures of isolate CW00B006C were stored in 15% glycerol at -80 °C. For each experiment, a cryovial of stock culture was warmed to room temperature in a 36°C water bath. A 50 µl aliquot of suspension from the vial was added to each 18 x 150 mm glass test tube containing 3 ml of appropriate medium. The test tubes of were incubated on an orbit shaker at 200 rpm for 24 h under ambient laboratory conditions (24 °C ± 3). Contents of tubes were used as 'seed inoculum'. The bacterial culture for inoculations was produced using 75 ml of sterile NGB or CWE in 250-ml Erlenmeyer flasks inoculated with 0.5 ml per flask of the appropriate 'seed inoculum'. Unless otherwise indicated, flasks were incubated on an orbit shaker at 200 rpm for 24 h under ambient laboratory conditions (24 °C ± 3). When stated, viable bacterial cell production was determined using the dilution plate count method. Serial dilutions (10x) were performed in dilution tubes containing 9 ml of sterile 0.01 M phosphate buffer, pH 7, and 100 µl of three appropriate dilutions were spread plated on nutrient glucose agar (NGA). Plates were incubated under ambient laboratory conditions for about 4 days. Colony forming units (CFU) per ml was determined by counting colonies on plates with 10-200 CFU per plate.

6.3.1.2. Plant production.

Chickweed seeds (7-10 seeds per pot) were sown in 10-cm diameter peat pots containing Sunshine Growing Mix (#1 SunGro Horticulture Canada Ltd., Seba Beach, Alberta, Canada). Seeded pots were placed in a greenhouse with 23/20±4°C day/night temperature, a 16 h photoperiod, an average light intensity of 300 $\mu Em^{-2}s^{-1}$, and an average relative humidity of 45-50%. After germination, seedlings were thinned to five plants per pot.

6.3.1.3. Inoculation.

Unless otherwise stated, three replicate pots containing chickweed seedlings at the 4-6-leaf stage were inoculated with culture suspension using an H-set airbrush (Paasche Airbrush Company, Harwood Heights, IL) at a pressure of 100 kPa. Unless otherwise stated, the culture suspension consisted of 10^9-10^{10} CFU/ml bacterial. The application volume of the culture suspension was 5 ml/pot. Immediately after spraying, pots were returned to the greenhouse and placed in a randomized block design for the remainder of the experiment. Disease severity was assessed using the 0-11 rating scale by Horsfall and Barrett (1945) one, two or three wks after spraying.

6.3.1.4. Effect of bacterial concentration on disease severity.

Culture was produced using NGB as described. Culture contents of flasks were centrifuged at 2440 rcf for 10 min to form a bacterial cell pellet (Sorvall RC-5B refrigerated superspeed centrifuge). The supernatant was decanted and the bacterial cell pellet from 60 ml of culture was resuspended in 30 ml of 0.01 M, pH 7, sterile phosphate buffer amended with 0.2% Silwet L-77®. The resulting spray solution was assumed to have a concentration of 10^{10} cells per ml and subsequent serial dilutions of the suspension with buffer and 0.2% Silwet L-77® were made to achieve spray inoculum with cell concentrations of about 10^9, 10^8, 10^7, 10^6 and 10^5 cells per ml. The viable bacterial concentration of each spray solution was then determined. The actual viable bacterial concentrations of each spray solution were very close to desired concentrations at 0.98×10^{10}, 1.8×10^9, 1.0×10^8, 1.8×10^7, 1.6×10^6, and 1.6×10^5 CFU/ml. Chickweed seedlings were inoculated with each spray solution. Control plants were inoculated with sterile 0.01 M phosphate buffer plus 0.2% Silwet L-77®. Efficacy of each solution was determined as described.

6.3.1.5. Effect of spray solution pH on disease severity.

Bacterial inoculum was produced using NGB as described. Culture contents of each flask were centrifuged at 2440 rcf for 10 min to form a bacterial cell pellet (Eppendorf 5810R centrifuge). Supernatant was decanted and the bacterial cell pellet from 40 ml of culture was resuspended in 20 ml of 0.1 M sterile phosphate buffer containing 0.2% Silwet L-77® with pHs from 6 to 8 at increments of 0.2. Chickweed seedlings were

inoculated with each spray solution having different pH. Control plants were inoculated with sterile 0.1 M phosphate buffer of the appropriate pH plus 0.2% Silwet L-77®. Efficacy of each solution was determined as described.

6.3.1.6. Effect of surfactant type and concentration on disease severity.

Inoculum was produced using NGB as described. Five organosilicone-based surfactants (Loveland Industries, Inc., Greeley, CO) were selected including Silwet L-77® (silicone-polyether copolymer 100%), Freeway (silicone-polyether copolymer and alcohol ethoxylates 100%), Phase (methylated esters of fatty acids and organosilicone surfactant fluids 100%), Tactic (synthetic latex plus organosilicone surfactant 62.5% and inerts 37.5%), and motion (polymethylsiloxane-copolymer and non-ionic surfactant 100%). Concentrations of 0.1, 0.2, and 0.3% were included for Silwet L-77® and Intac while surfactant concentrations of 0.1, 0.2, 0.3, and 0.5% were included for remaining three surfactants. Surfactants were added to bacterial inoculum just prior to spraying. Efficacy of different surfactants was determined as described with a treatment consisting of NGB and surfactant serving as a control for each bacterial treatment.

6.3.1.7. Effect of repeat application on disease severity.

Inoculum was produced using NGB as described. Replicate pots containing chickweed seedlings at the 4-6-leaf stage were initially inoculated with bacterial solution produced as described and amended with 0.2% Silwet L-77® just prior to spraying. Immediately after spraying, pots were returned to the greenhouse and placed in a randomized block design. Inoculated common chickweed seedlings were then resprayed with bacterial inoculum, produced as described and amended with 0.2% Silwet L-77 just prior to spraying, 2, 6, and 9 days after initial application. One replicate set of pots with no repeat spray application was maintained. Control treatments with NGB and surfactant were included for all bacterial treatments. Disease severity was assessed beginning 1 wk after the initial bacterial application.

6.3.1.8. Effect of bacterial growth medium and inoculum growth stage on efficacy.

Inoculum was produced using both CWE and NGB as described with incubation of bacterial inoculum in each medium for 24, 48 or 72 hours. Inoculation of flasks was

staggered at 24 h intervals such that all inoculum was ready at the same time. Viable bacterial cell concentration of each treatment was determined as described prior to amendment with 0.15% Silwet L-77® for spray application. Control treatments consisting of sterile medium and wetting agent were included for both NGB and CWE. Disease severity was assessed as described.

6.3.1.9. Effect of bacterial growth stage and chickweed growth stage on disease severity.

Inoculum was produced using CWE as described. Two trials to study the impact of chickweed growth stage on disease severity were conducted. For trial 1, flasks of bacterial inoculum were incubated 24 hours as described. For trial 2, flasks of bacterial inoculum were incubated either 24 or 48 hrs prior to determination of viable bacterial cell concentration as described and amendment with 0.15% Silwet L-77® for spraying. Replicate pots of chickweed seeded and grown under greenhouse conditions as described for one (cotyledon growth stage), two (2-4 leaf stage), three (6-8 leaf stage) or four weeks were inoculated with bacterial or control solution. Spray solution for control treatments consisted of tap water and 0.15% Silwet L-77 for trial 1 and uninoculated CWE and 0.15% Silwet L-77 for trial 2. Disease severity for both trials was assessed as described.

6.4.1.10. Infectivity and disease severity on Group 2 herbicide resistant chickweed.

Inoculum was produced using NGB as described. Group 2 herbicide resistant chickweed seeds were obtained from the ARC Weeds Research Laboratory. Previous tests indicate that the average survival rate of the herbicide resistant seed set with 1x and 2x Ally (metsulfuron methyl) application was 73.5 and 35.2%, respectively (O'Donovan, et al., 1994). The chickweed seed set used for all other experiments was included as a herbicide susceptible positive control. Both herbicide resistant and susceptible seedlings were produced as described and seedlings of both seed sets at the 6-8 leaf stage were inoculated with the bacterial inoculum using the previously described method. Differences in infectivity and disease severity between the herbicide resistant and the herbicide susceptible populations were compared as described.

6.3.2. Efficacy under field conditions

6.3.2.1. Experiments conducted in outside pots.

Efficacy of *B. andropogonis* for the control of chickweed under natural conditions was first evaluated in large pots set outside. Two experiments were conducted, one in 2001 (July 24 – September 14) and the second in 2002 (May 30 – July 31). Results from the two experiments were not pooled because the variances were not homogeneous according to Bartlett's test (Gomez & Gomez, 1984). However, a similar trend was observed and the results from 2001 are presented.

For both experiments, chickweed plants were produced in 53-cm-diameter fiber pots filled with soil collected from a field at the ARC, Vegreville experimental site (loam, pH 6.7, 42% sand, 40% silt, and 18% clay) and placed outside on the ground where building walls would provide some shade. Chickweed seeds were sown in the pots 3-4 weeks prior to spraying by sprinkling seeds evenly over the soil surface, then lightly sprinkling soil over the seeds. Pots were watered as necessary using an outside tap throughout the experiment. Bacterial inoculum was prepared as described using NGB and 24 h incubation of flasks for experiment 1 and CWE and 48 h of incubation for experiment 2. Inoculum at a cell concentration of 3.0×10^9 CFU/ml for experiment 1 and 5.0×10^9 CFU/ml for experiment 2, with a surfactant concentration of 0.15% Silwet L-77® for both experiments was used. A control treatment with NGB and wetting agent was included for experiment 1, while a control with tap water and wetting agent was included for experiment 2. Chickweed plants were sprayed at approximately the 6-leaf stage. For experiment 1, pots with chickweed seedlings at the 6-leaf stage were brought inside and placed, 3 replicate pots at a time, in the spray chamber. Each pot was sprayed with 100 ml of appropriate solution using an airbrush at 100 kPa. After spraying, pots were immediately placed back outside. For experiment 2, a 2L Spray-Doc compressed air sprayer (Gilmour Manufacturing Co., Somerset, PA, USA) was used to spray about 115 ml per pot without moving them from their outside location. Plants were assessed for symptoms using the 0-11 rating scale by Horsfall and Barrett (1945) when they became most apparent, about 2 weeks after spraying. Daily weather conditions during the experiments were recorded including maximum and minimum temperatures (°C), precipitation (mm), sunlight period (h), and average radiation (watts/m^2). Dry weight per pot was determined 4 weeks after spraying by cutting aerial parts at the soil level, drying

in a paper bag for 48 h at 70°C, and weighing. The dry weight (DW) data were expressed as percent reduction in biomass compared with biomass of controls and calculated using the formula: Dry weight reduction (%) = (DW in control – DW in inoculated treatment)/DW in control x 100. The experiments were arranged in a completely randomized design. Data was analyzed by SAS ANOVA.

6.3.2.2. Experiments conducted in field plots.

Efficacy of CW00B006C for the control of chickweed at different growth stages under natural conditions was further evaluated in field plots. Two field trials were conducted at the ARC Vegreville experimental site in 2002: trial one was conducted from May 27 – July 29 and trial 2 from June 24 – August 30. Results from the two trials were not pooled because the variances were not homogeneous according to Bartlett's test (Gomez & Gomez, 1984). However, a similar trend was observed between trials and the results from trial 1 are presented.

A mini-plot (0.5 m x 1.0 m) design with a 0.5 m boarder of bare earth surrounding each split plot was used (Figure 5.9). The main experimental factor was chickweed growth stage, with seeding dates at 1 wk intervals (June 17, June 24, July 2, and July 8, 2002 for trial 1), while the subplot factor was bacterial application rate (0 or 10^9 CFU/ml). For each of four replicate 0.5 m x 1.0 m plots, one half (0.5 m x 0.5 m) was sprayed with control solution and the other half with bacterial inoculum. For each seeding date, 2 g of chickweed seed were evenly sown over the surface of a 0.5 m x 1 m plot and lightly covered with soil until seeds were no longer visible (about 2 mm deep). Unless soil was wet at the surface from recent rains, all plots were watered using a watering can with a sprinkler nozzle. The soil surface was kept moist at all times until seeds germinate. Weeds other than chickweed in the boarder areas of plots were hand-removed. Bacterial inoculum was produced using CWE with 48 h incubation in flasks as described. When the youngest chickweed seedlings reached the cotyledon growth stage, bacterial inoculum or tap water (control) plus 0.15% Silwet L-77® was sprayed on each subplot at an application volume of 150 ml per 0.5m x 0.5m subplot using a 2L Spray-Doc compressed air sprayer (Gilmour Manufacturing Co., Somerset, PA, USA). Efficacy was assessed beginning 1 wk after spraying as described. Twenty-eight (28) days after treatment, the aboveground dry biomass of chickweed was determined. All percentage

data were arc sine-transformed before analysis (Gomez & Gomez, 1984). Experiments were analyzed with an analysis of variance considering the effect of main and subplot factors and their interaction, using PROC ANOVA procedure in SAS (SAS Institute Inc., 1990). Treatment means were separated using LSD at the 5% level of significance.

6.4. Results and discussions

6.4.1. Efficacy under greenhouse conditions

6.4.1.1. Effect of bacterial concentration on disease severity.

Disease was observed on chickweed seedlings with all bacterial concentration treatments two weeks after spraying. Disease severity significantly increased when the bacterial concentration was increased (Figure 5.1). Disease severity was also significantly greater 3 wks versus 2wks after spraying. About 65% disease severity was observed with the bacterial concentration of 10^9 and 10^{10} CFU/ml three weeks after spray.

6.4.1.2. Effect of spray solution pH.

No damage was observed on control plants. Spray solution pH did not affect weed control efficacy in the pH range of 6-8.

6.4.1.3. Effect of surfactant type and concentration on disease severity.

Effectiveness of chickweed control with *B. andropogonis* was affected by surfactant type ($P > 0.001$), concentration ($P > 0.003$), and their interaction ($P > 0.001$). Of the five surfactants studied, Silwet L-77® significantly enhanced the bacterial efficacy for the control of chickweed while the remaining surfactants did not show any efficacy enhancement (Figure 5.2). There was no significant difference in efficacy among treatments of 0.1%, 0.2%, and 0.3% Silwet L-77®. However, higher concentration of Silwet L-77® in control treatments showed higher phytotoxicity to chickweed seedlings. Findings in this study suggest that the best concentration of Silwet L-77® to be used with the bacterial inoculum is between 0.1% and 0.2%.

6.4.1.4. Effect of repeat application on disease severity.

Repeat application of the bacterial inoculum enhanced disease severity on chickweed seedlings caused by *B. andropogonis*, but this enhancement varied with the time interval

of the second application (Figure 5.3). Significant enhancement of disease severity on chickweed was observed with the repeat application nine days after the first application. Therefore, repeat application could be a method to improve the efficacy of *B. andropogonis* as a bioherbicide against chickweed. Effect of application frequency and time interval should be further investigated.

6.4.1.5. Effect of bacterial growth medium and inoculum growth stage on disease severity.

Disease severity was significantly affected by the addition of chickweed extract to the culture medium ($P > 0.0071$) and the inoculum growth stage ($P > 0.0176$) (Figure 5.4). Similar patterns were observed for the percent dry weight reduction ($P > 0.036$ for the addition of chickweed extract and $P > 0.048$ for the inoculum growth stage). Inoculum growth stage at 48 h resulted in the greatest disease severity and percent dry weight reduction. Both disease severity and percent dry weight reduction caused by the bacterium cultured in CWE medium for 48 h were significantly greater than that caused by the bacterium cultured in NGB.

6.4.1.6. Effect of inoculum growth stage and chickweed growth stage on disease severity.

Chickweed seedlings at different growth stages responded differently to the fungus *B. andropogonis*. Disease severity was increasing with older chickweed growth stage (Figure 5.5). The highest disease severity was observed with the 4-wk-old chickweed seedlings. Disease severity in 4-wk-old chickweed seedling caused by *B. andropogonis* was significantly greater than that in 1-, 2-, or 3-wk-old chickweed seedlings (Figure 5.5). However, there was no difference in percent dry weight reduction among chickweed seedlings at different growth stages treated with *B. andropogonis*.

Inoculum growth stage significantly affected disease severity in chickweed seedlings caused by *B. andropogonis* (Figure 5.6). Inoculum cultured for 48 h caused greater disease severity in chickweed seedlings at all four growth stages than inoculum cultured for 24 h. For both inoculum growth stages, disease severity was increasing with older chickweed growth stage. However, there was no difference in percent dry weight reduction of chickweed seedlings at each of four growth stages treated with inoculum cultured for 24 h or 48 h. Statistically, no interaction between inoculum growth stage and chickweed growth stage was observed ($P > 0.7218$). Therefore, inoculum growth stage at

48 h and chickweed growth stage at 4 wk (6-8 leaf) are optimal for *B. andropogonis* to cause disease in chickweed.

6.4.1.7. Infectivity and disease severity on Group 2 herbicide resistant chickweed.

Herbicide resistant seedlings were also susceptible to the bacterial treatment. There was no difference in disease severity between the herbicide resistant and the herbicide susceptible seedlings when treated with the same bacterial inoculum. Therefore, the use of *B. andropogonis* provides a new approach to combat Group 2 herbicide resistance in chickweed and may mitigate the development of herbicide resistance.

6.4.2. Efficacy under field conditions.

6.4.2.1. Experiments conducted in outside pots.

Severe disease was observed on chickweed seedlings 1 wk after bacterial treatment. Two wks after inoculation, disease severity was rated as 75% (Figure 5.8). Seventy two percent dry weight reduction was obtained 4 weeks after inoculation. Disease incidence reached 100% (Figure 5.9). Maximum and minimum temperatures (°C), precipitation (mm), sunlight period (h), and average radiation (watts/m^2) during this experiment are shown in Fig. 5.7.

6.4.2.2. Experiments conducted in field plots.

Severe disease was observed on chickweed seedlings 1 wk after bacterial treatment. Disease incidence reached 100% (Figure 5.12). Chickweed seedling at different growth stages exhibited different disease severity (Figure 5.11). The greatest disease severity was observed on the oldest chickweed seedlings (about 80%), the least disease severity on the youngest chickweed seedlings (about 38%). However, there was no significant difference in dry weight reduction, ranging from 57.6% to 79.5%, among the different growth stage treatments (Figure 5.11). Maximum and minimum temperatures (°C), precipitation (mm), sunlight period (h), and average radiation (watts/m^2) during this experiment are shown in Figure 5.10. Higher temperature and less precipitation might contribute to greater efficacy of *B. andropogonis* for control of chickweed. If this was true, this bacterium possesses excellent potential as a bioherbicide in western Canada due to the common dry conditions.

6.5. Literature Cited

1. Horsfall, J.G. and R.W. Barrett .1945. An improved grading system for measuring plant diseases. Phytopathol. 35:655.
2. O'Donovan, J.T., Jeffers, G.M., Maurice, D., and Sharma, M.P. 1994. Investigation of a chlorsulfuron-resistant chickweed weed [*Stellaria media* (L.) Vill.] population. Can. J. Plant Sci. 74: 693-697.
3. Watson, A.K. and L.A. Wymore. 1990. Identifying limiting factors in the biocontrol of weeds. Pages 305-316 in New Directions in the Biological Control: Alternatives for Suppressing Agricultural Pest and Disease. Alan R. Liss, Inc.

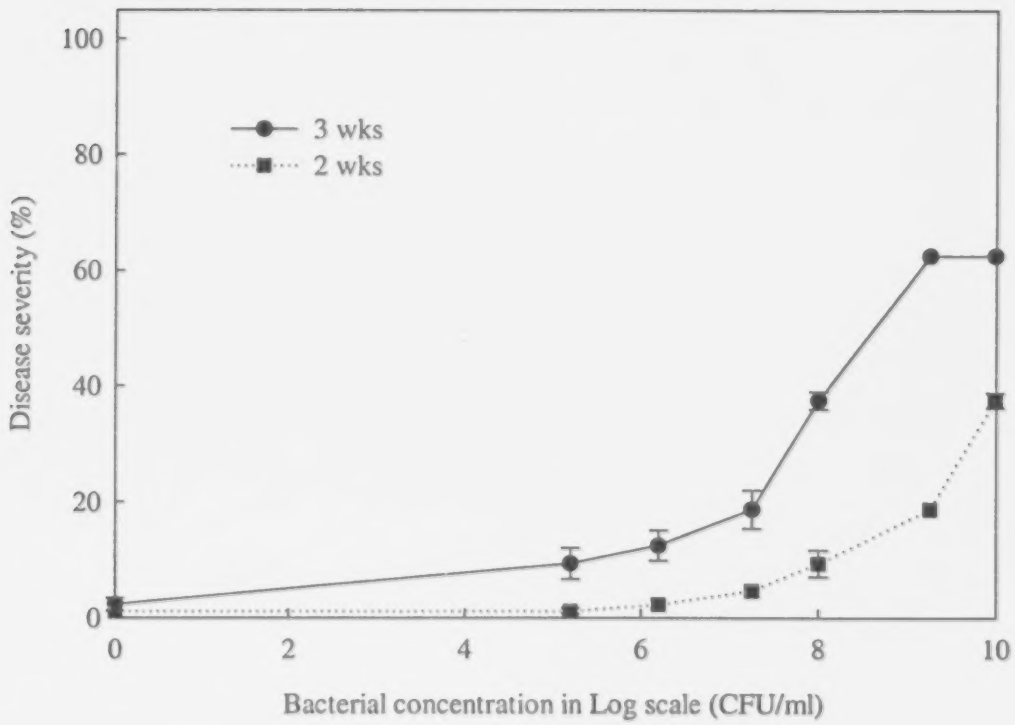

Figure 6.1 Effect of CW00B006C bacterial concentration on the disease severity of chickweed.

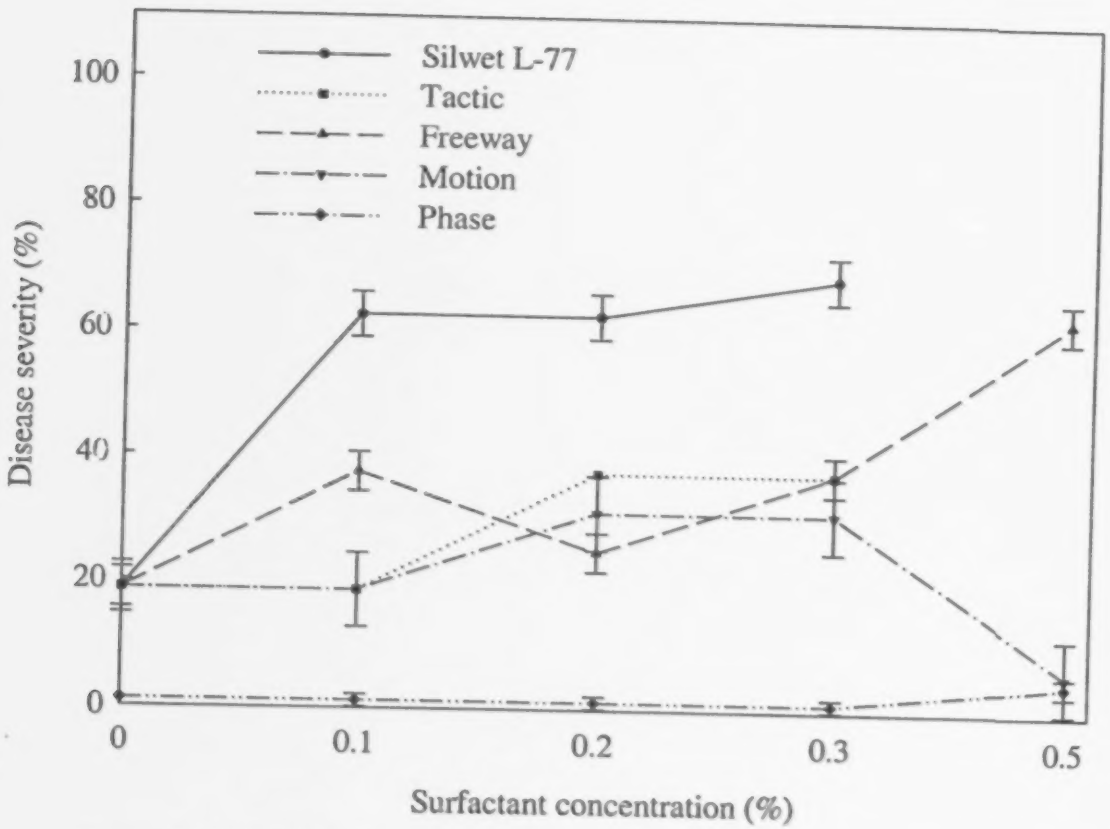

Figure 6.2 Effect of surfactant type and concentration on disease severity of chickweed caused by *Burkholderia andropogonis*

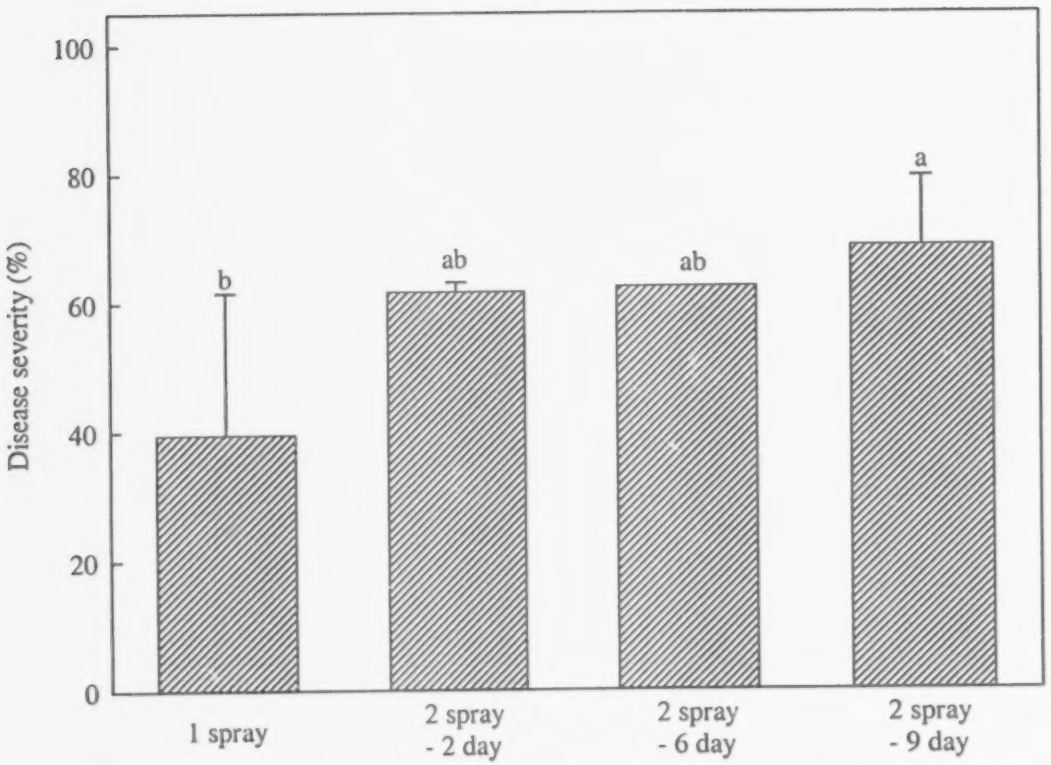

Figure 6.3 Effect of repeat application on disease severity of chickweed caused by *Burkholderia andropogonis*

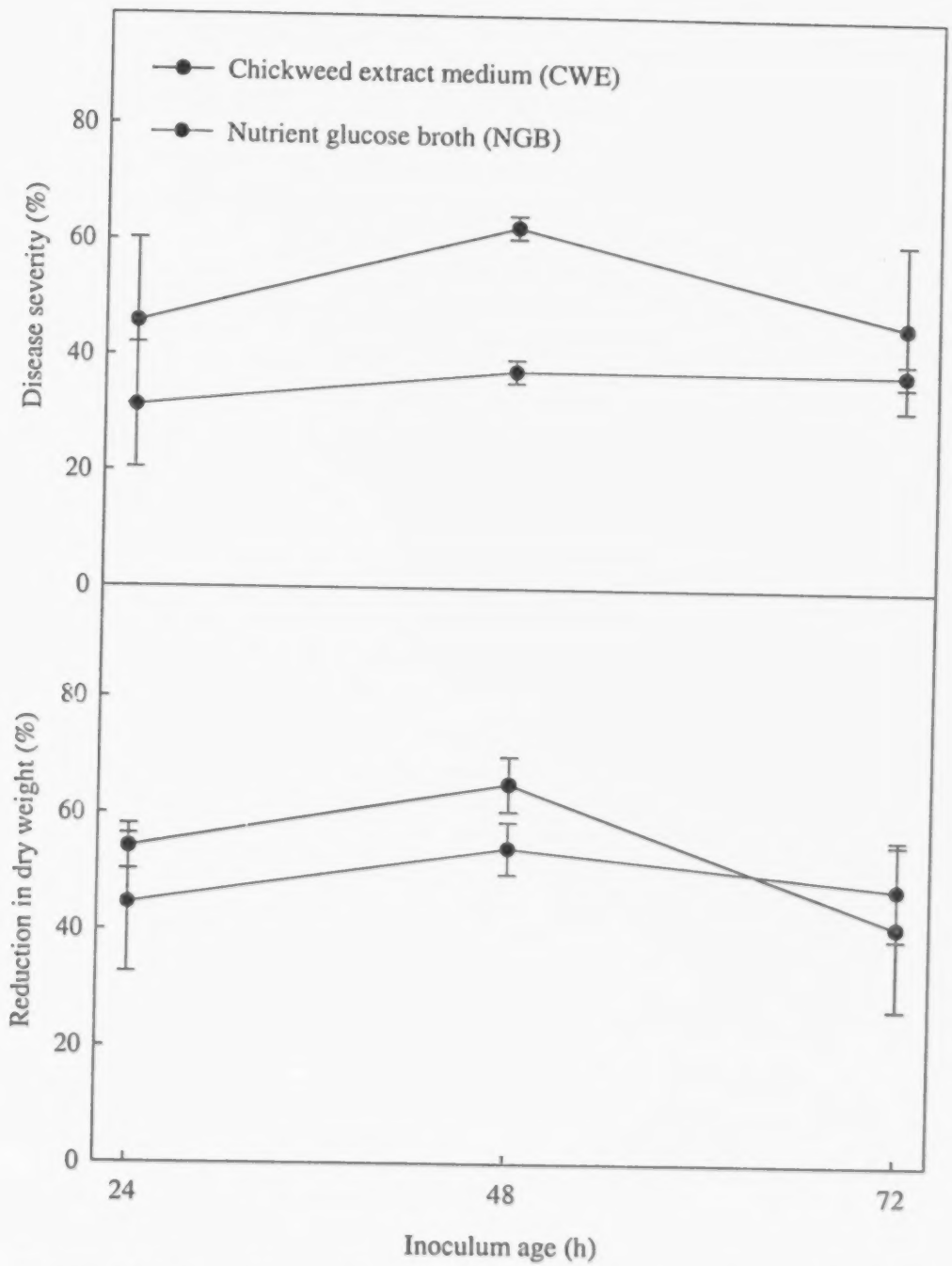

Figure 6.4 Effect of bacterial growth medium and inoculum growth stage on disease severity of chickweed caused by *Burkholderia andropogonis*

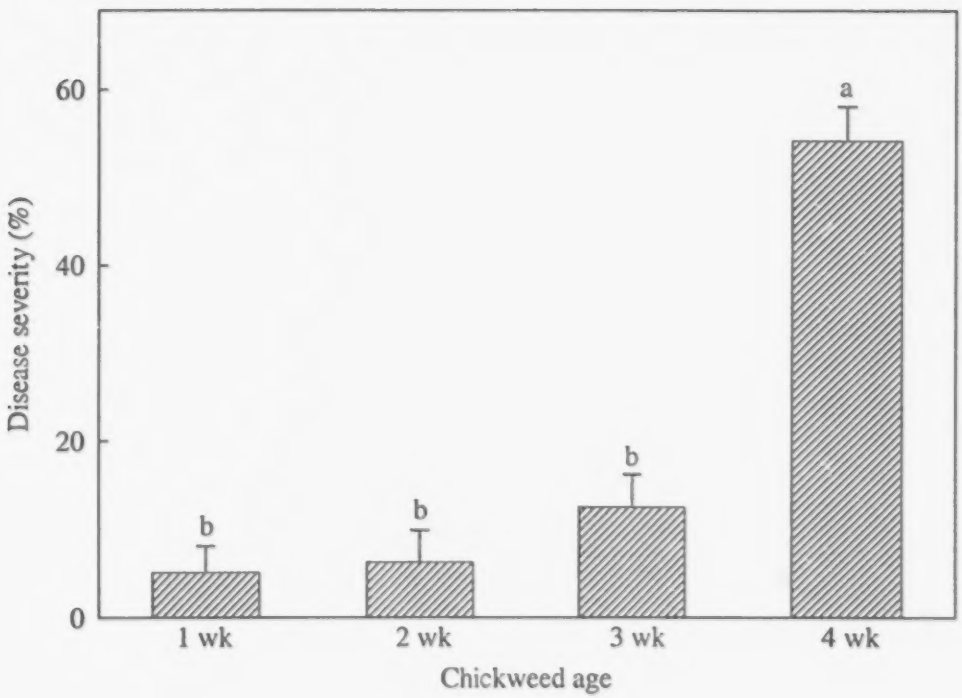

Figure 6.5 Effect of chickweed growth stage on disease severity of chickweed caused by *Burkholderia andropogonis*

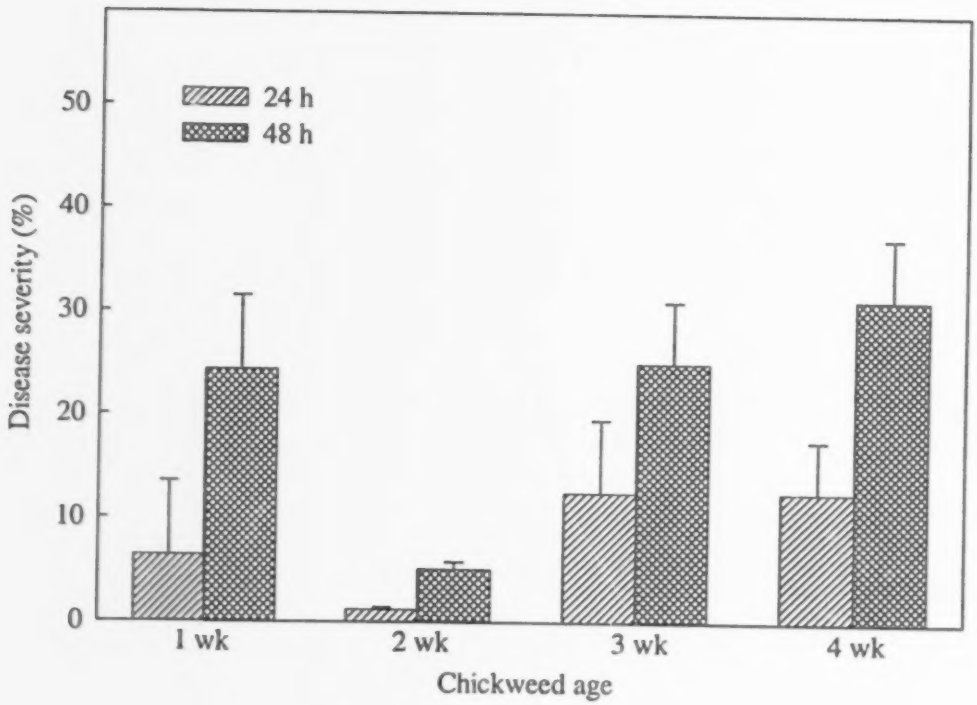

Figure 6.6 Effect of bacterial growth stage and chickweed growth stage on disease severity of chickweed caused by *Burkholderia andropogonis*

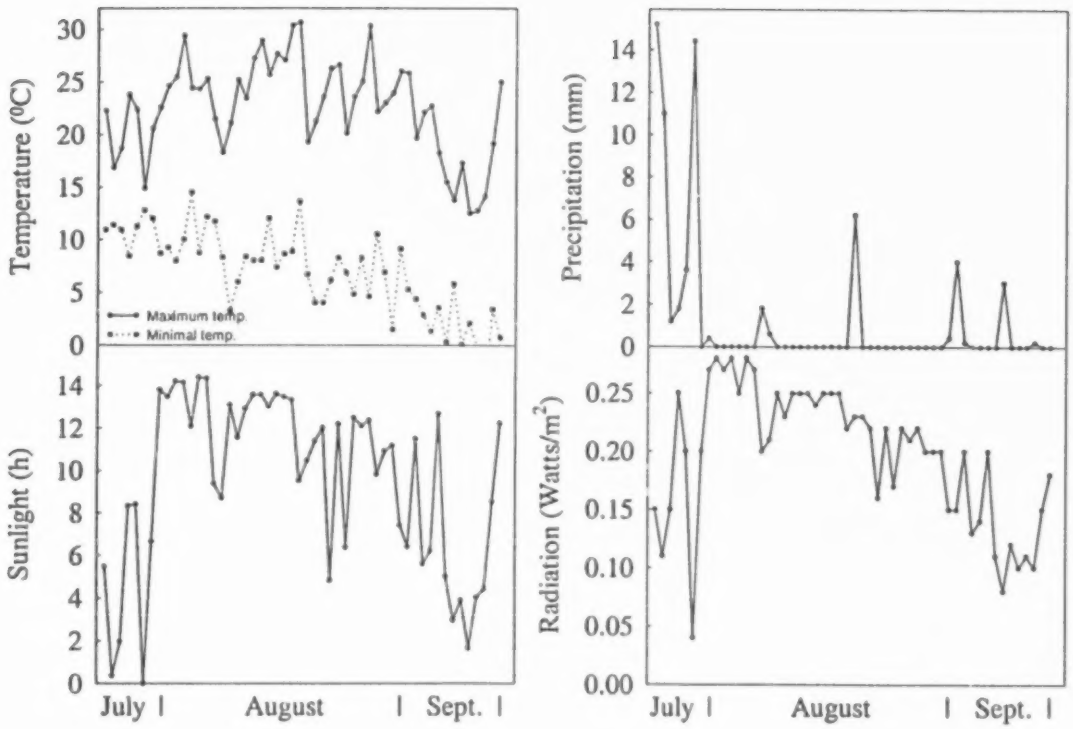

Figure 6.7 Daily weather conditions from July 24 to September 14, 2001 recorded during the field evaluation of chickweed control with bacterial strain CW00B006C. (A) Maximum and minimum temperatures (°C), (B) Precipitation (mm), (C) Sunlight period (h), and (D) Average radiation (watts/m^2).

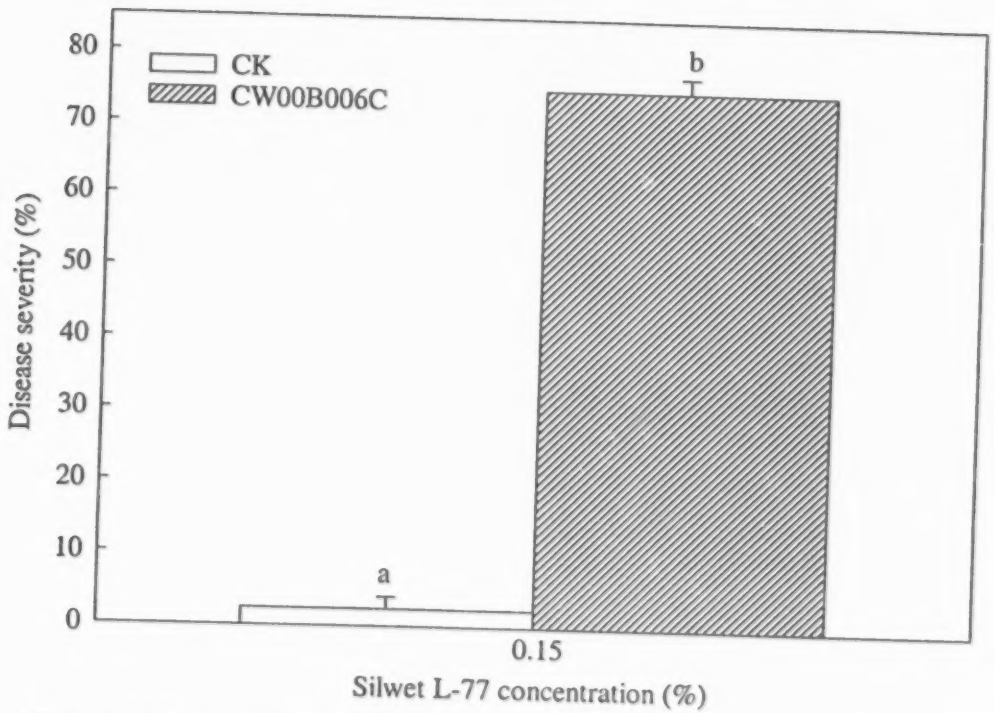

Figure 6.8 Disease severity on chickweed grown in outside pots caused by *Burkholderia andropogonis*.

Figure 6.9 Infectivity of *Burkholderia andropogonis* on chickweed under field conditions.
Left – Treated, Right – Control

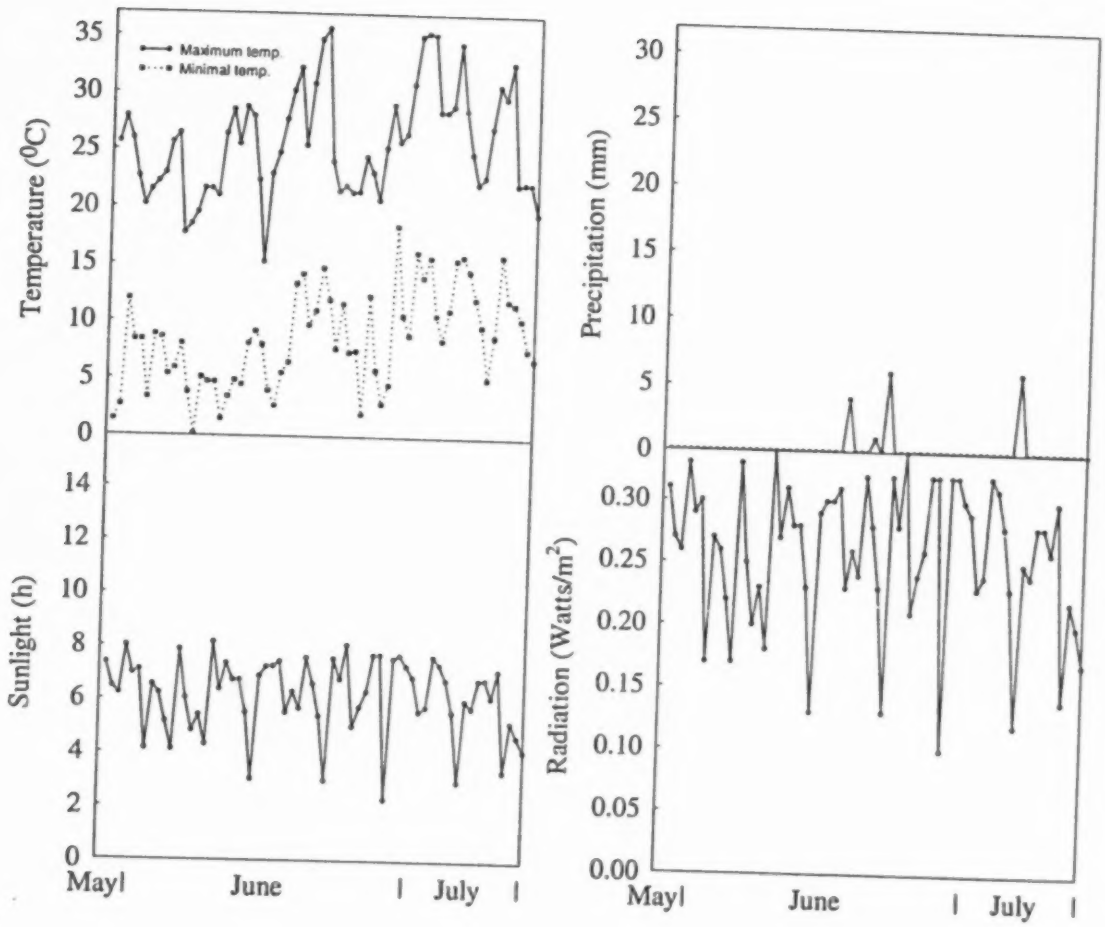

Figure 6.10 Daily weather conditions from May 27 to July 29, 2002 recorded during the field evaluation of chickweed control with bacterial strain CW00B006C. (A) Maximum and minimum temperatures (°C), (B) Precipitation (mm), (C) Sunlight period (h), and (D) Average radiation (watts/m^2).

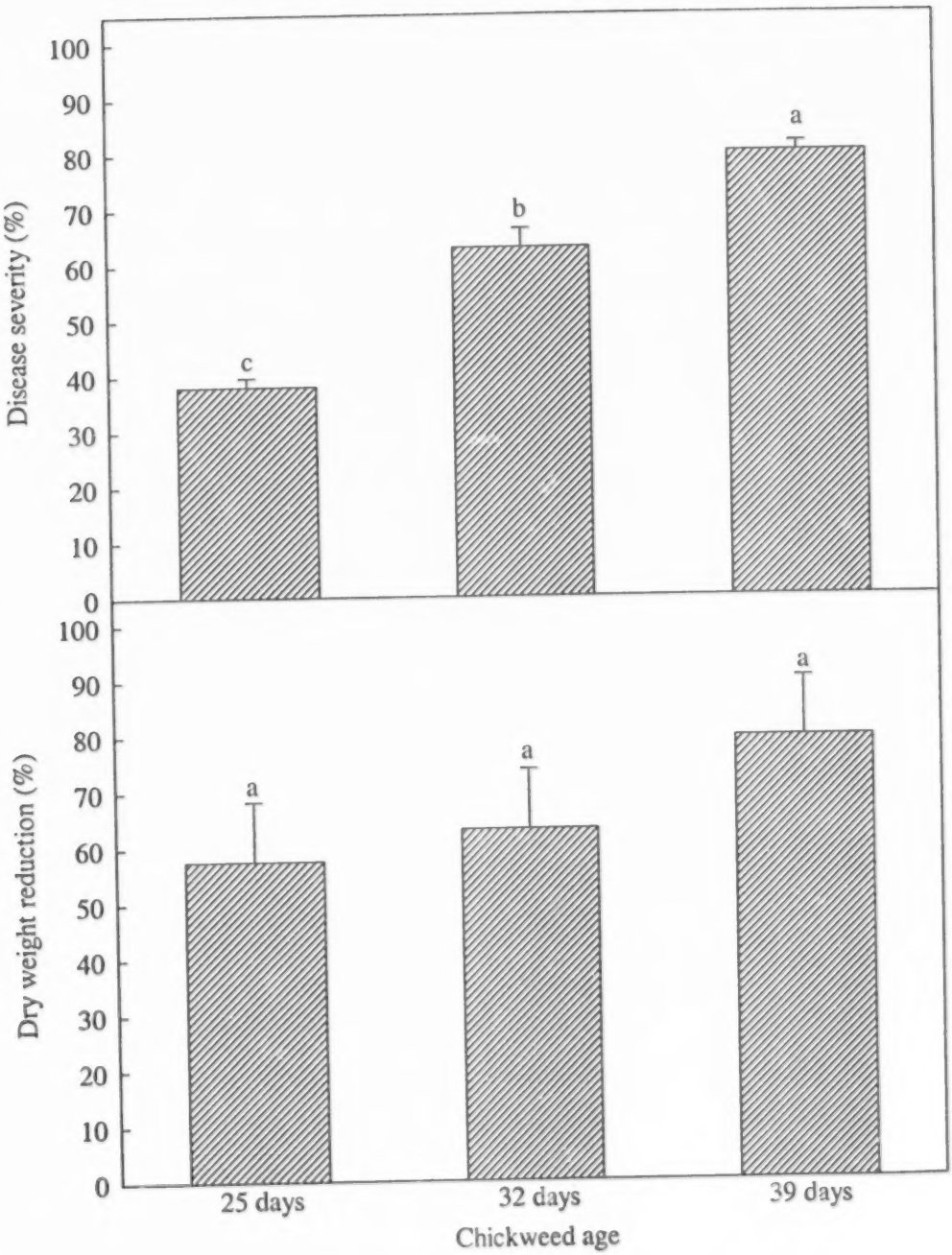

Figure 6.11 Disease severity and dry weight reduction of chickweed caused by *Burkholderia andropogonis*

Figure 6.12 Chickweed control with the bacterium *Burkholderia andropogonis* (A) Field trial conducted in 2002 and (B) Detailed weed control performance in one plot.

Chapter 7 Natural products

7.1 Abstract

Cell-free culture filtrates caused symptoms similar to those caused by the bacterial cells, indicating the potential of using fermentation broth alone, or combination with bacterial cells for chickweed control.

7.2. Introduction

Production of two amino acids, rhizobitoxine and hydroxyreonine, has been considered as a general feature of *Burkholderia andropogonis* (Mitchell and Frey, 1988). However, potential for the use of these two amino acids to control weeds have not been extensively evaluated. Recently, several studies have demonstrated that amino acids and/or peptides can be used as biologically based herbicides (Duke et al., 2002). For example, five dipeptides from corn gluten hydrolysate can control both grasses and broadleaf weeds, with alanylalanine being the most active (Liu and Christians, 1994). Thus, the objective of this study is to determine if the natural products produced by *Burkholderia andropogonis* have any herbicidal activity against chickweed.

7.3 Materials and Methods

A cryovial of stock culture was warmed to room temperature in a 36°C water bath. Streak plates were then made on King's medium B agar Petri plates (KB; 20 g proteose peptone #3 (Difco), 1.5 g. K_2HPO_4, 1.5 g $MgSO_4 \cdot 7H_2O$, 15 ml glycerol, 15 g Bacto Agar (Difco), 1 L distilled water) using the stock culture. After 24 h incubation under ambient laboratory conditions on KB agar, a loopfull of cells was transferred to each 18 x 150 mm glass test tube containing 3 ml yeast-glucose broth (YGB; 5 g yeast extract (Difco), 5 g glucose, 0.3 g $MgSO_4$, 3.9 g K_2HPO_4, 3.8 g KH_2PO_4, 1 L distilled water) at a pH of 6.8. The test tubes were incubated on an orbit shaker at 200 rpm for 24 h under ambient laboratory conditions (24 °C ± 3). Contents of tubes were combined as 'seed inoculum' and 0.5 ml of the 'seed inoculum' was used to inoculate each 500 ml flask containing 150 ml of filter sterilized, pH 6.8, Hoitink & Sinden chemically defined medium (HS; 10 g glucose, 3.6 g K_2HPO_4, 4.1 KH_2PO_4, 0.2 g $MgSO_4$, 1 g NH_4Cl, 1 mg biotin, 1 L distilled water). Flasks were incubated on an orbit shaker at 200 rpm for 6 d under ambient

laboratory conditions (24 °C ± 3). Contents of flasks were combined and viable bacterial cell production was determined using the dilution plate count method as previously described. Culture was centrifuged 10 minutes at 2440 rcf (Eppendorf 5810R centrifuge), supernatant was poured from each tube and passed through a 0.22 µm bottle-top vacuum filter to remove all bacterial cells.

7.3.1. Heat stability of the cell-free culture filtrates

A sample of cell-free filtrate was autoclaved 30 minutes at 121°C to determine the effect of heat on the bacterial phytotoxins.

7.3.2. Effect of the cell-free culture filtrates on chickweed root growth

A sample of both the autoclaved and unautoclaved cell-free filtrate was used for a laboratory assay designed to study the effects of the bacterial phytotoxins on chickweed seed germination and seedling root growth. A Whatman #1 filter paper was placed in replicate glass Petri Plates. Solutions of autoclaved and unautoclaved cell-free filtrates at 100, 50, 25, 12.5 and 6.25% of the initial concentration were prepared using sterile distilled water. A control of sterile, distilled water was also prepared. A 5 ml aliquot of the appropriate treatment was pipetted into each of three replicate Petri plates. Using forceps, four chickweed seeds, as used for all other experiments, were placed in each plate. Plates were placed on the laboratory bench under ambient laboratory conditions (21 ± 2°C) for incubation and arranged in a randomized block design. Seed germination, seedling root length (mm), and seedling appearance were assessed after 1 wk.

7.3.3. Diseases caused by the cell-free culture filtrates

Remaining autoclaved and unautoclaved cell-free filtrate was then shell frozen in Fast-Freeze Flasks (Labconco), freeze-dried, and used to inoculate chickweed seedlings in the greenhouse. Once dry, samples were stored at –80°C until use. Freeze-dried samples were resuspended in distilled water at a concentration of 10 times that of the original by gentle shaking. For the concentrated, unautoclaved cell-free filtrate, one 15 ml sample was amended to contain 0.1% Silwet L-77®, a second to contain 0.2% Silwet L-77®, a third was amended to contain 0.5% Tween 80 as a wetting agent and a fourth sample was left unamended. The autoclaved sample was amended to contain 0.2% Silwet L-77®.

Control treatments with freeze-dried HS medium and wetting agent were also prepared. Three replicate pots containing chickweed seedlings at the 6-8-leaf stage were inoculated for each treatment using an H-set airbrush (Paasche Airbrush Company, Harwood Heights, IL) at a pressure of 100 kPa. Plants were grown as previously described. Immediately after spraying, pots were returned to the greenhouse and placed in a randomized block design for the remainder of the experiment. Plants were monitored for any symptoms for 3 wks following spraying

7.4. Results and Discussions

Cell free culture filtrates from HS medium did not affect chickweed seed germination. However, they caused completely chlorotic chickweed seedlings and significantly inhibited root growth (Figure 7.1). The activity of root growth inhibition and chlorosis from the cell-free culture filtrates was not affected by heat, i.e. autoclave vs. unautoclave, suggesting the natural products produced by CW00B006C are heat stable.

In the greenhouse studies, approximately 4-6 days after spraying, slight to moderate chlorosis was visible on new leaves of chickweed sprayed with all autoclaved and unautoclaved cell-free filtrates amended with Tween-80 and Silwet L-77®. There was no difference in disease severity on chickweed seedlings between heat treatments, confirming the natural products produced by CW00B006C was heat stable. New leaves on plants showing symptoms were pale yellow or white. No symptoms were seen on chickweed sprayed with cell-free filtrate without wetting agent or on control treatments. Thus, there is potential to develop the bacterial natural products as biologically based herbicide for the control of chickweed. Further study is required to characterize the compounds in the cell-free culture filtrates and compare to the two reported amino acids.

7.5. Literature Cited

1. Duke, S.O., F.E. Dayan, A.M. Rimando, K.K. Schrader, G. Aliotta, A. Oliva, and J.G. Romagni. 2002. Chemicals from nature for weed management. Weed Science 50:138-151.
2. Liu, D.L.Y. 1994. Isolation and identification of root-inhibiting compounds from corn gluten hydrolysate. Journal of Plant Growth Regulation 13:227-230.

3. Mitchell, R.E. and E.J. Frey. 1988. Rhizobitoxine and hydroxythreonine production by *Pseudomonas andropogonis* strains, and the implications to plant disease. Physiol. and Mol. Plant Pathol. 32:335-341.

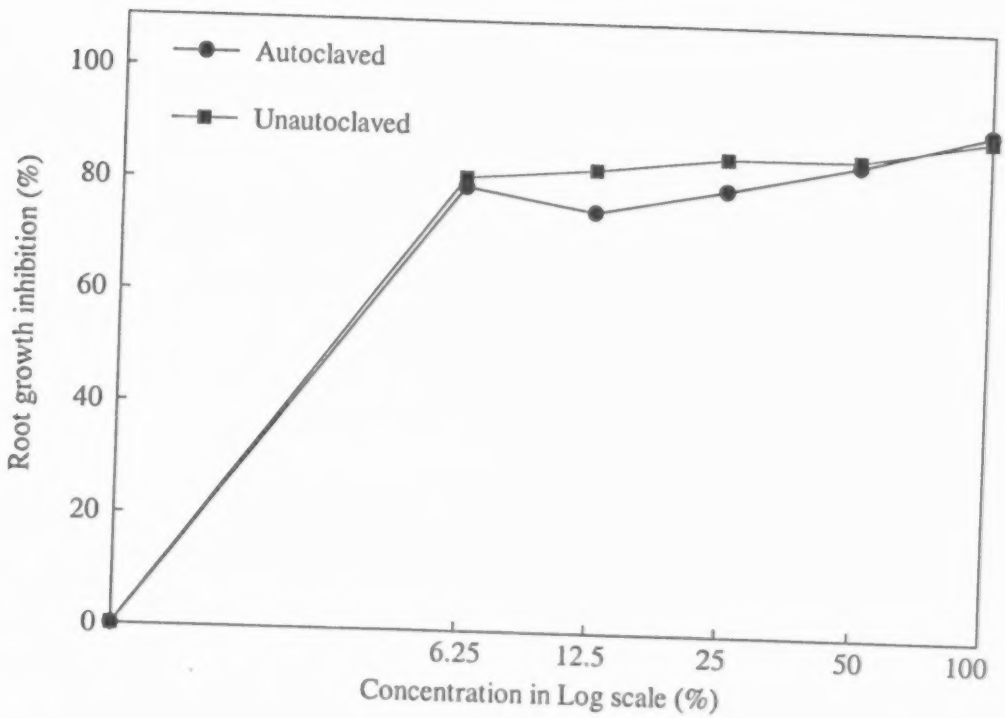

Figure 7.1 Effect of cell free culture filtrates of CW00B006C on root growth of chickweed seedlings.

Part III Deliverables

Patents:

1. United States Paten Application No. 20020177528: Biocontrol of weeds
2. Canadian Patent Application No. 2,325,215: Biocontrol of weeds.
3. Provisional Canadian Patent: Chickweed bioherbicide

Referred journal articles:

1. Zhang, W., M. Sulz, and K.L. Bailey. 2002. Evaluation of *Plectosporium tabacinum* for control of herbicide-resistant and herbicide-susceptible false cleavers. *Weed Science* 50: 79-86.
2. Zhang, W., M. Sulz, K.L. Bailey, and D.E. Cole. 2002. Effect of Epidemiological Factors on the Impact of the Fungus *Plectosporium tabacinum* on False Cleavers (*Galium spurium*). *Biocontrol Science and Technology* 12:183-194.
3. Zhang, W., M. Sulz, and K.L. Bailey. 2001. Growth and sporulation of *Plectosporium tabacinum*. *Canadian Journal of Botany* 79: 1297-1306.
4. Zhang, W., K. McKee, M. Sulz, T. Mykietiak, X. Li, D.E. Cole and K. L. Bailey. 2003. Infection process of *Plectosporium tabacinum* on false cleavers (*Galium spurium*). *Biocontrol Science and Technology* 13: (in press).

Proceedings paper:

1. Zhang, W. and K.L. Bailey. 2000. Biological control of cleavers (*Galium spurium* and *G. aparine*) with pathogenic fungi – exploration and discovery. *In* N.R. Spencer, eds. Proceedings of the X International Symposium on Biological control of Weeds. Bozeman, MT, pp.95-101.